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Lee et al.

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(54) **METHOD FOR DETECTING INTERACTIONS BETWEEN TWO AND MORE BIOLOGICAL MACROMOLECULES**

33/542 (2013.01); G01N 33/557 (2013.01);
C07K 2319/01 (2013.01); C07K 2319/60
(2013.01)

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(58) **Field of Classification Search**
CPC G01N 33/53
See application file for complete search history.

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(56) **References Cited**

(73) Assignee: **Korea Basic Science Institute**, Daejeon (KR)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 76 days.

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(21) Appl. No.: **13/733,687**

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(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation-in-part of application No. 12/547,043, filed on Aug. 25, 2009, now abandoned.

Foreign Application Priority Data

Nov. 12, 2008 (KR) 10-2008-0112354

(51) **Int. Cl.**

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C12Q 1/02 (2006.01)

G01N 33/50 (2006.01)

G01N 33/542 (2006.01)

G01N 33/557 (2006.01)

C12N 15/10 (2006.01)

(52) **U.S. Cl.**

CPC C12Q 1/025 (2013.01); C12N 15/1055 (2013.01); G01N 33/5035 (2013.01); G01N

(57) **ABSTRACT**

Disclosed is a novel method for detecting interactions of biomolecules. More particularly, the disclosed method includes (a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material; (b) detecting the distribution of the first construct and the second construct in the cell. the present invention provides a method capable of detecting bindings and interactions occurring in a living cell in real time, and a method for screening a material that alters the binding and the interaction. The method of the present invention overcomes the disadvantages including inaccuracy and complexity of existing biomaterial interaction detection techniques. By labeling both constructs to promote accuracy, the present invention provides a novel real-time, antibody-free analysis.

5 Claims, 17 Drawing Sheets
(15 of 17 Drawing Sheet(s) Filed in Color)

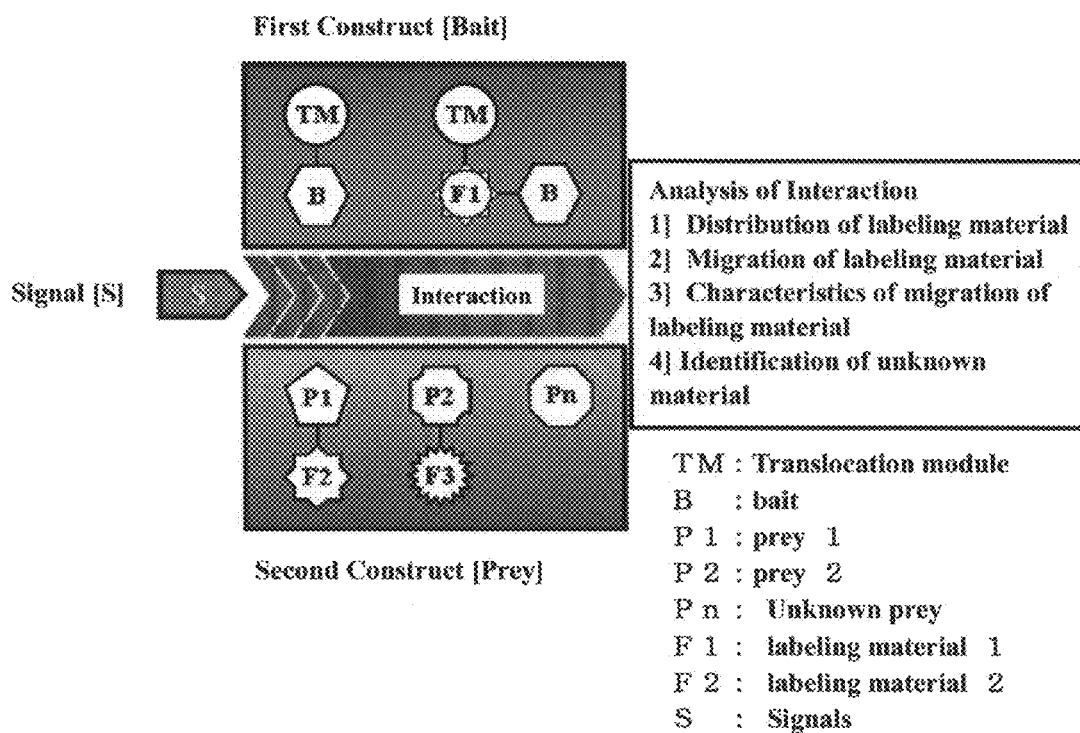
FIG 1

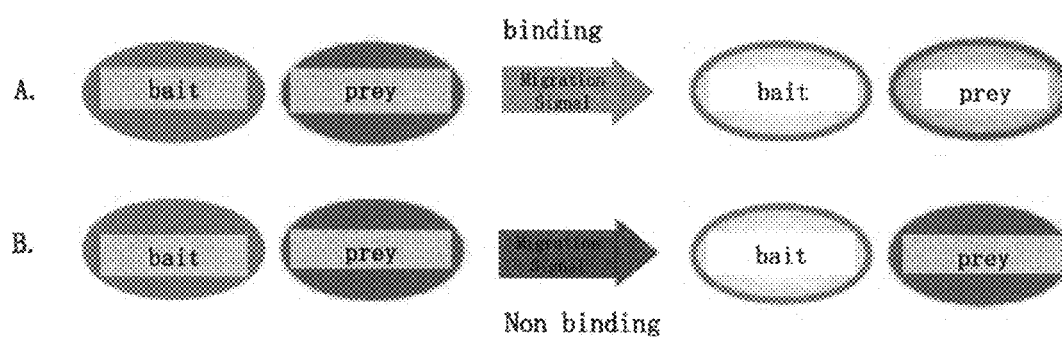
FIG 2

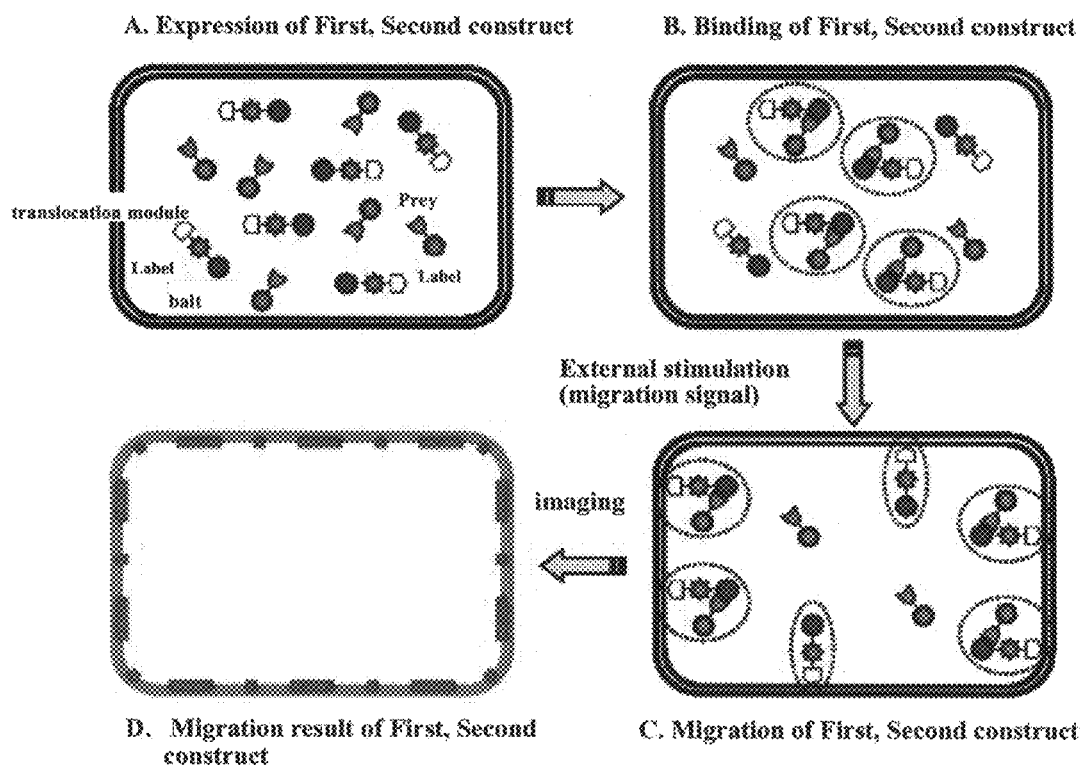
FIG 3

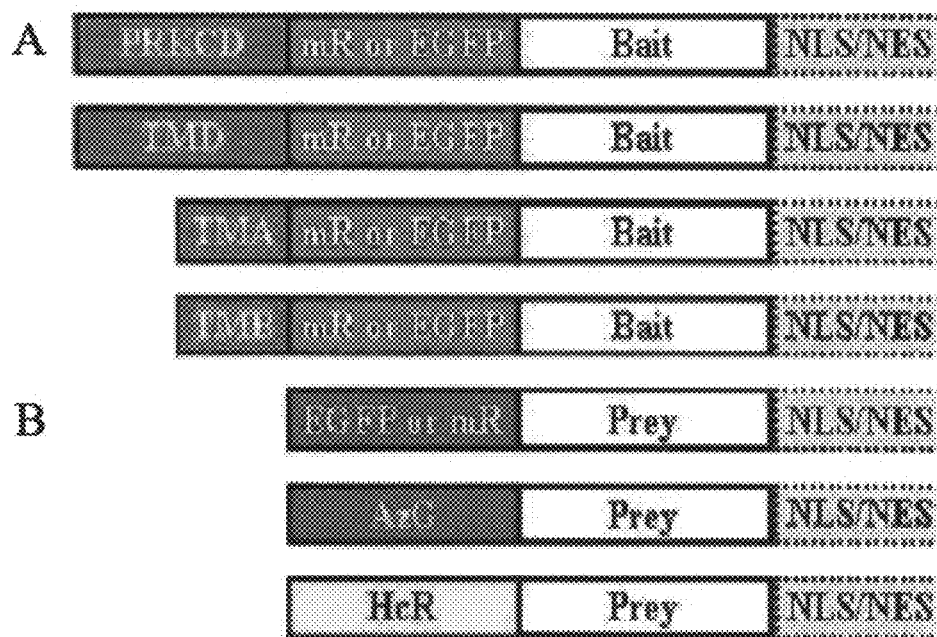
FIG 4

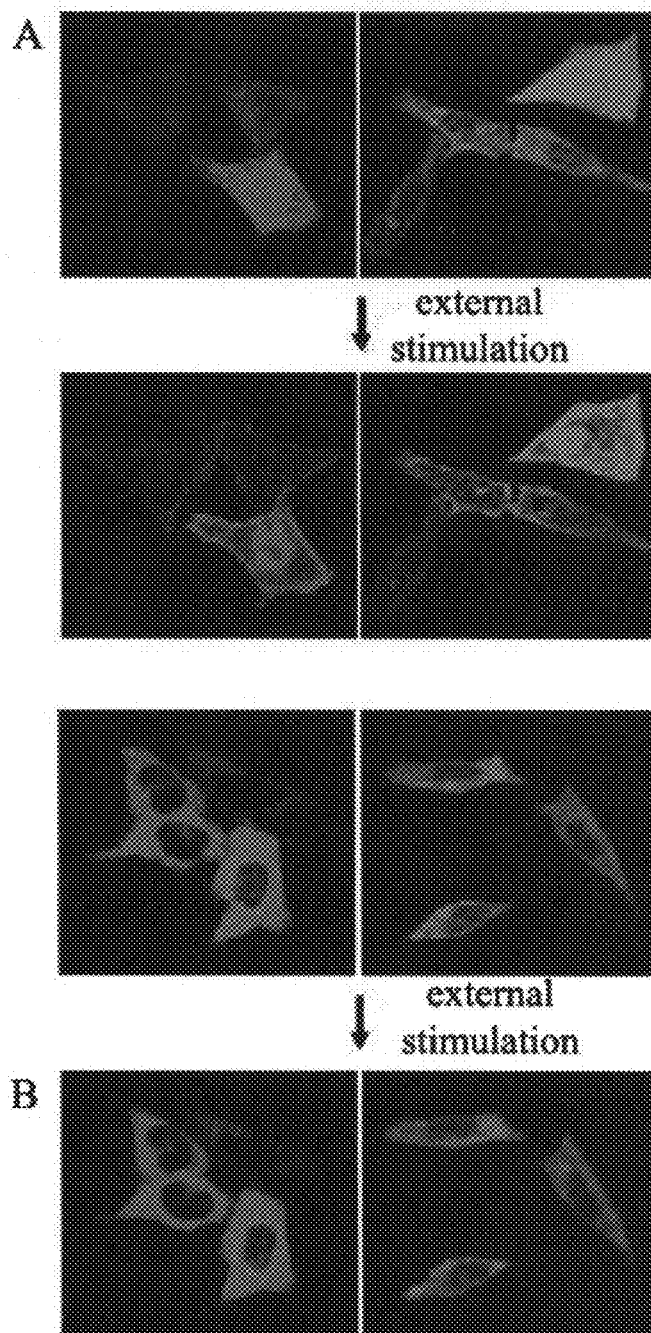
FIG 5

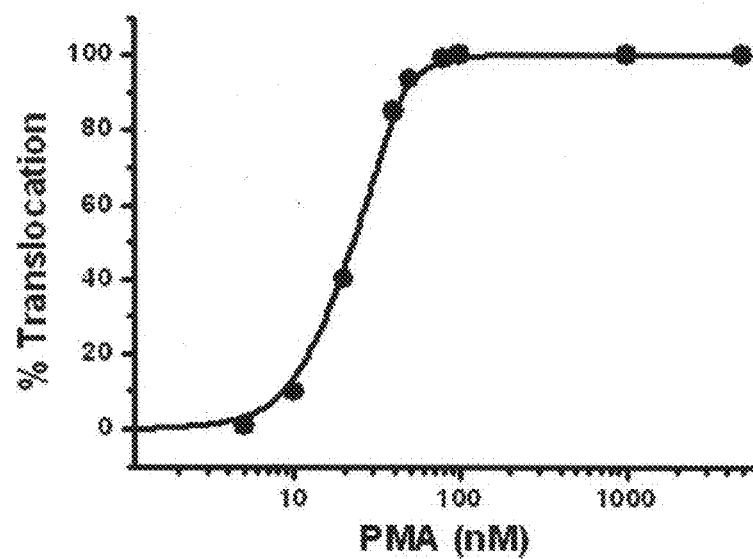
FIG 6

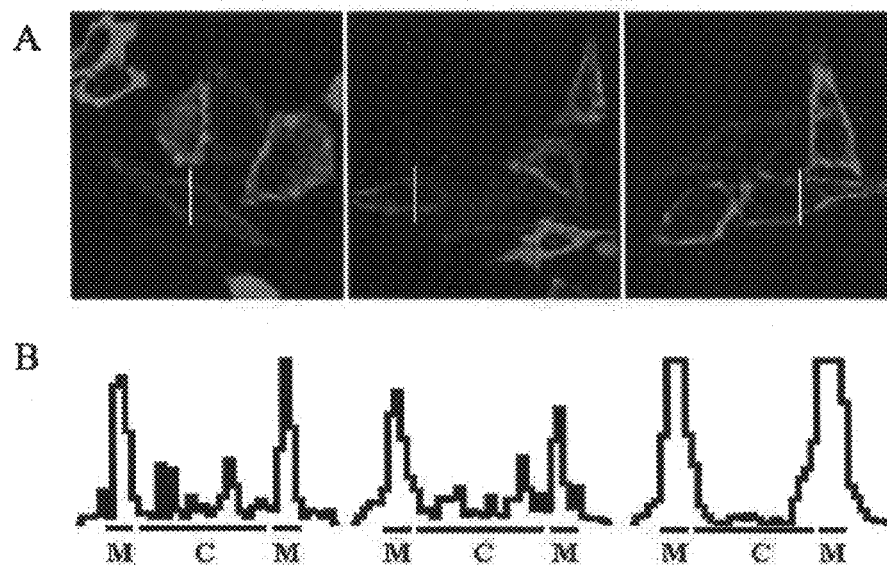
FIG 7

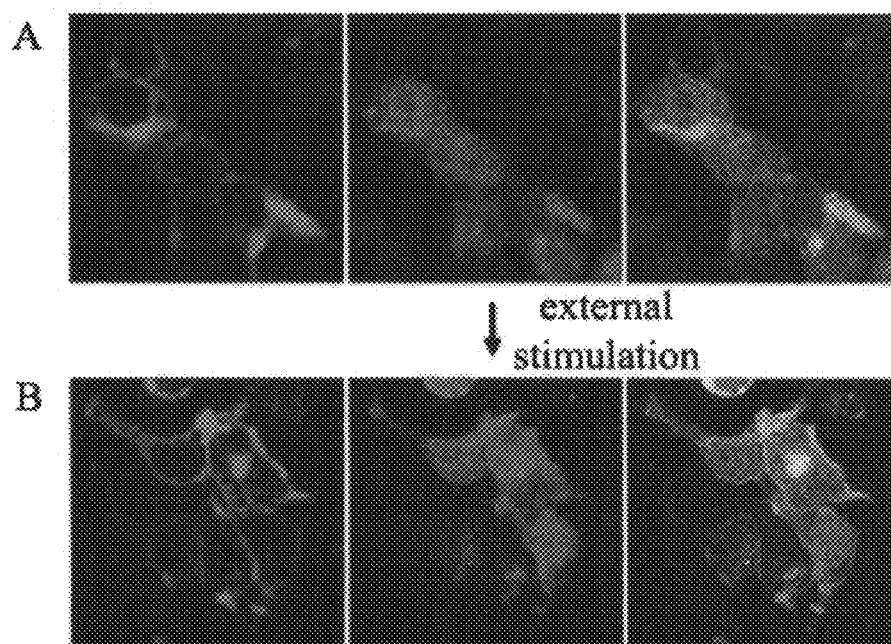
FIG 8

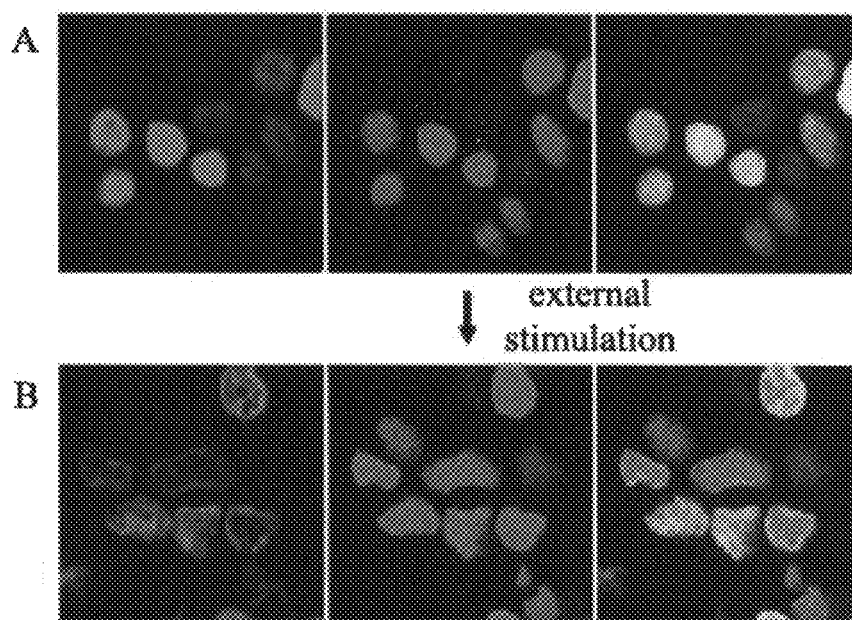
FIG 9

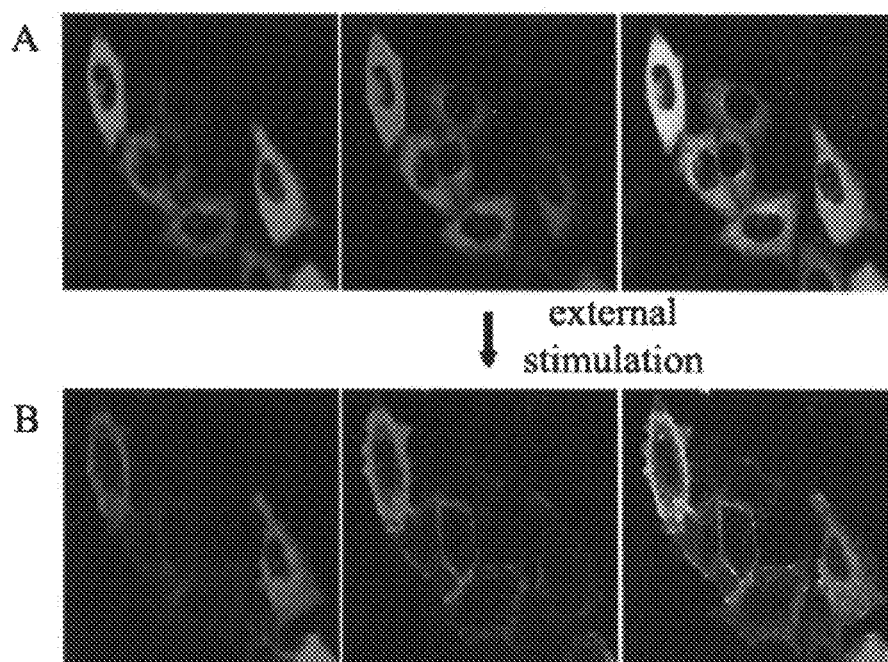
FIG 10

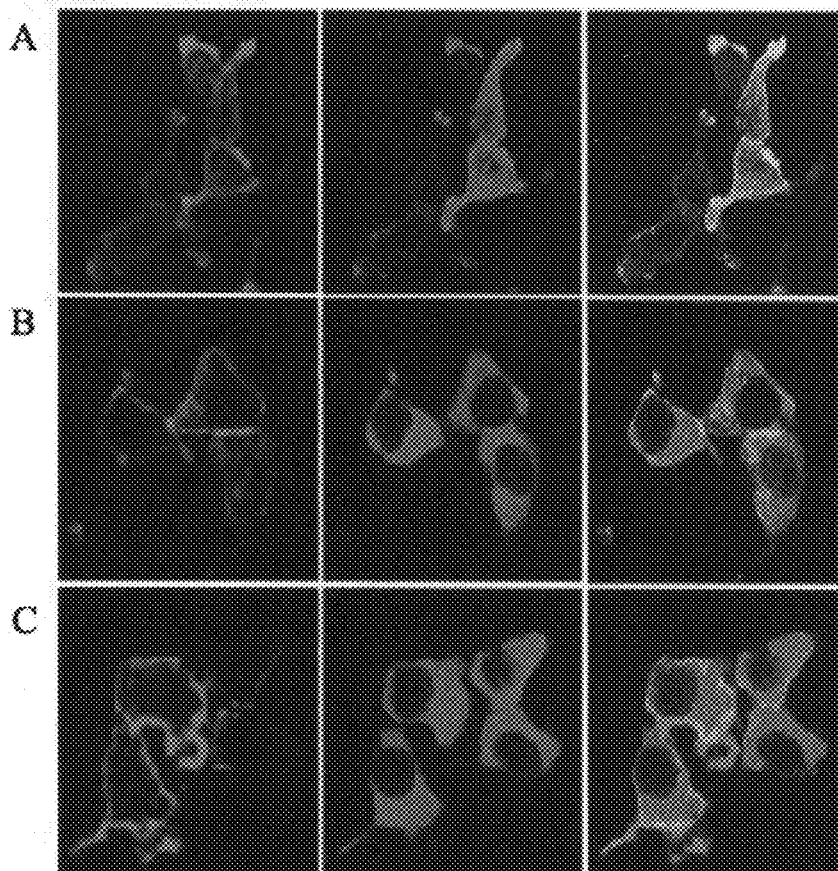
FIG 11

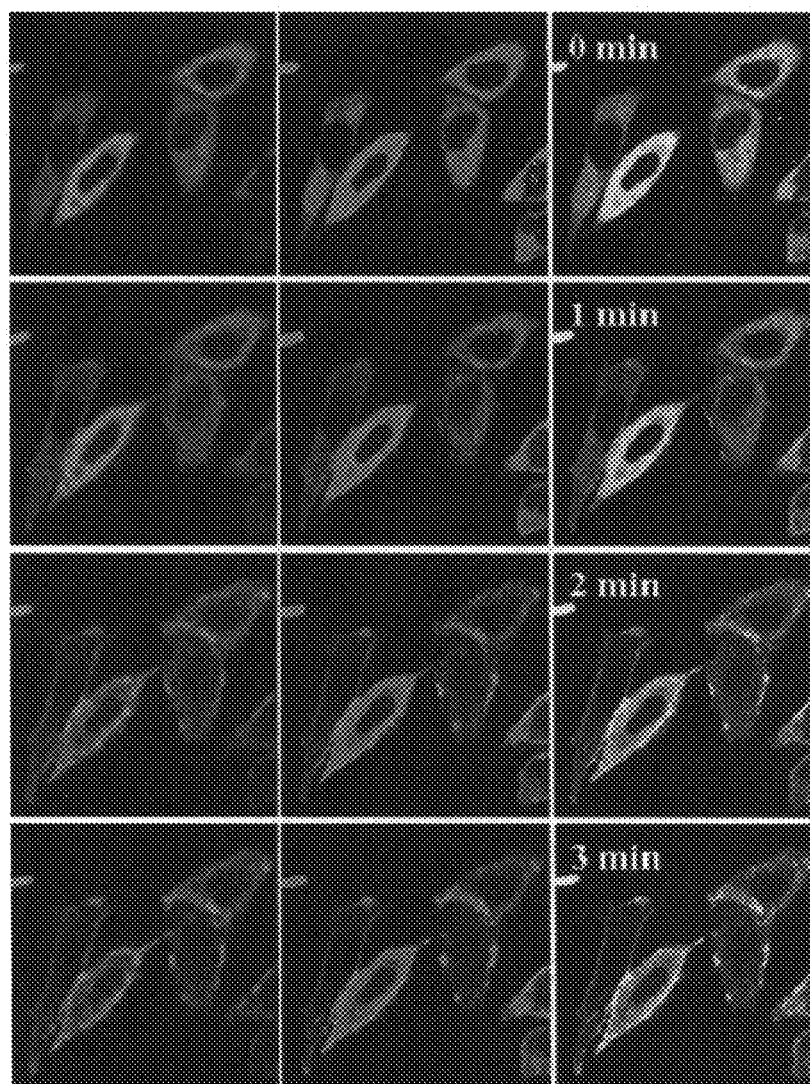
FIG 12

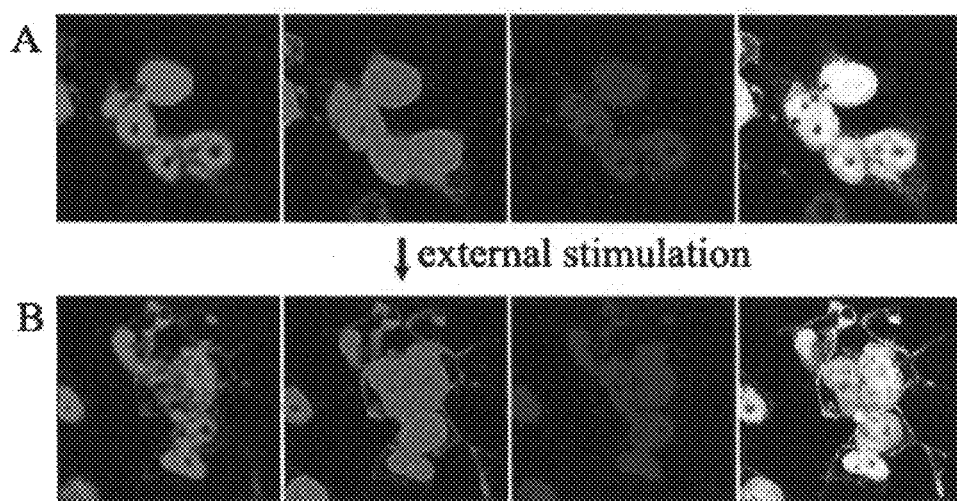
FIG 13

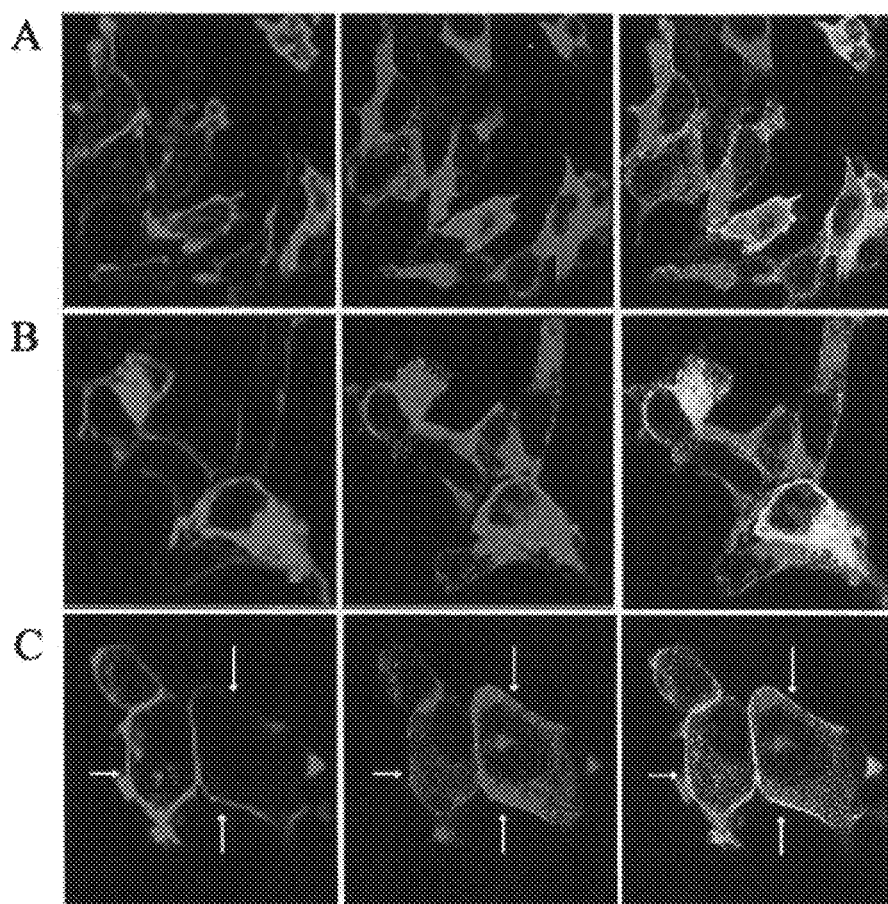
FIG 14

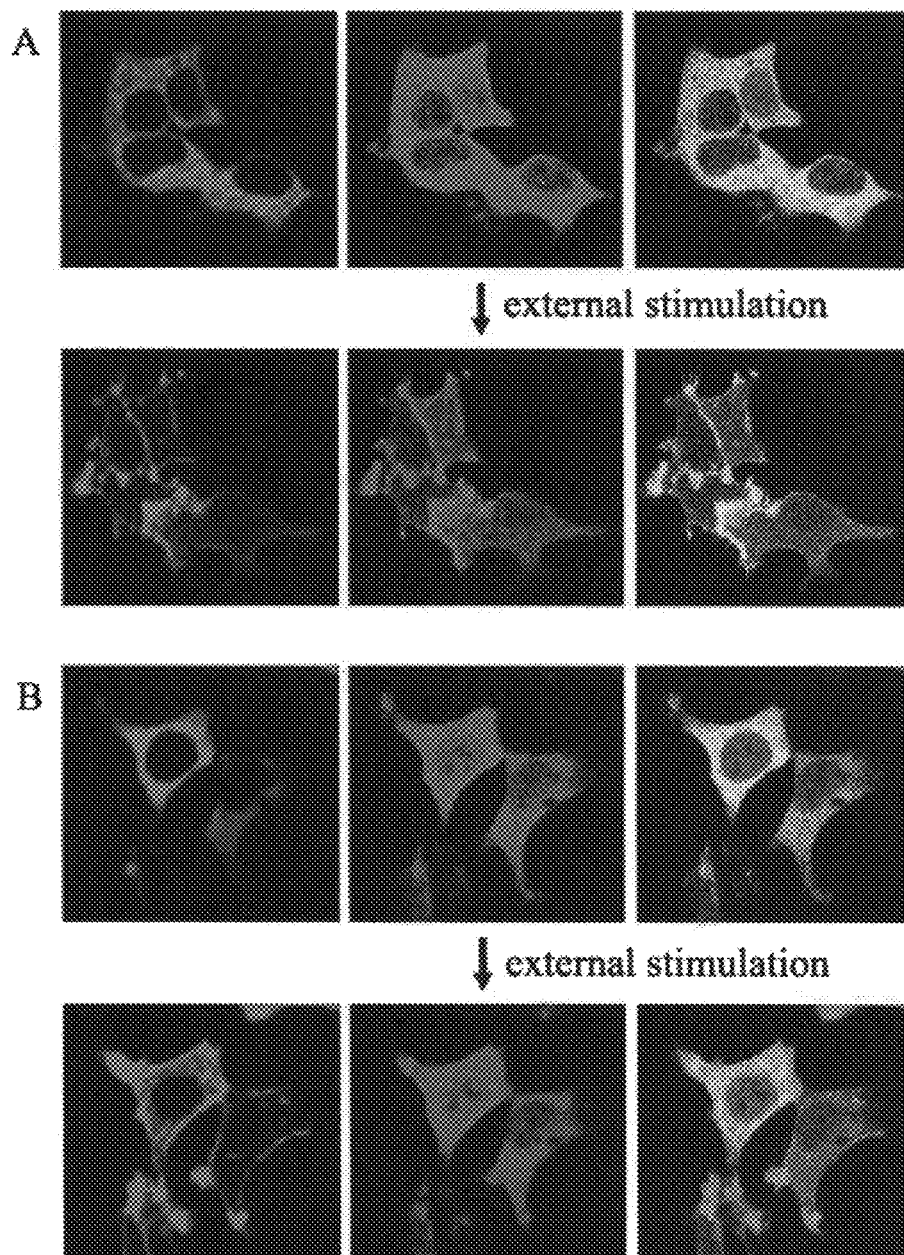
FIG 15

FIG 16

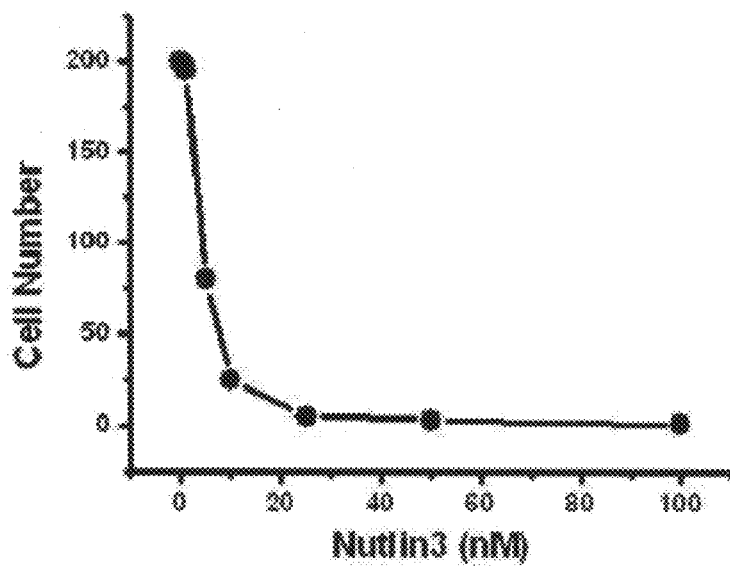
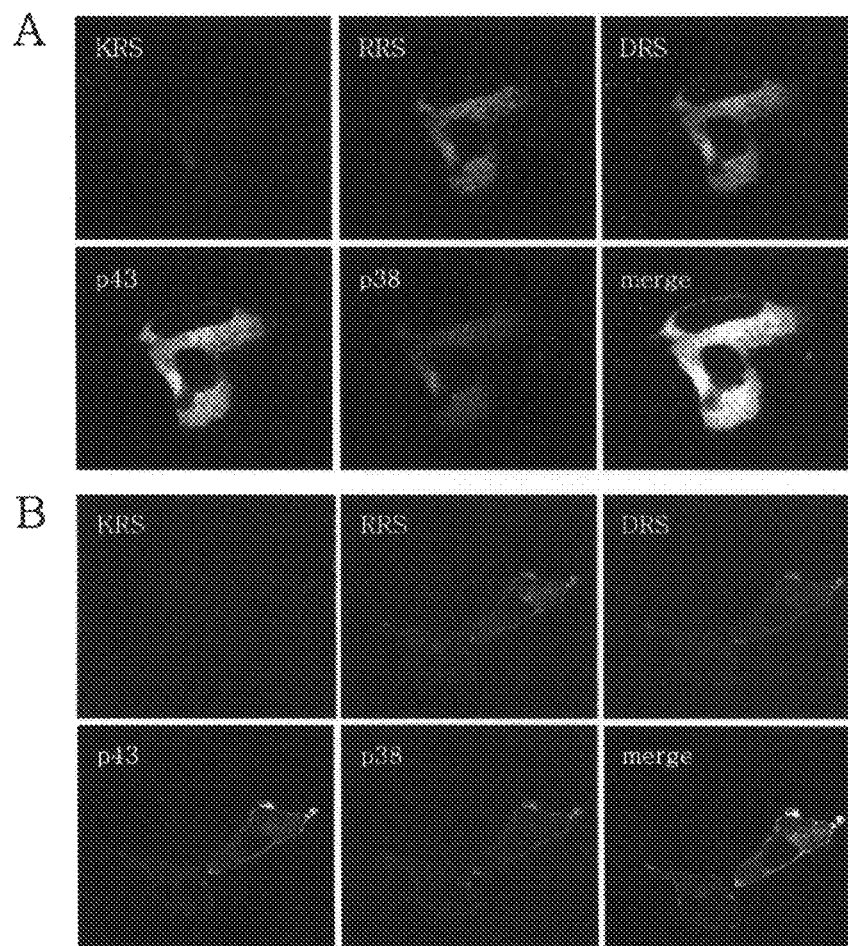


FIG 17

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METHOD FOR DETECTING INTERACTIONS BETWEEN TWO AND MORE BIOLOGICAL MACROMOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a Continuation-In-Part application of U.S. application Ser. No. 12/547,043 filed Aug. 25, 2009, now abandoned, which claim priority to Korean Application No. 10-2008-0112354 filed Nov. 12, 2008, patented Mar. 12, 2010, which applications are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jan. 2, 2013, is named 84724-CIP-307161_ST25.txt and is 57,284 bytes in size.

TECHNICAL FIELD

Disclosed is a novel method for detecting interactions of biomolecules. More particularly, the disclosed method includes (a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material; (b) detecting the distribution of the first construct and the second construct in the cell.

BACKGROUND

Growth, differentiation, migration, death and the like of cells are mediated by macromolecular interactions such as protein-protein or protein-nucleic acid interactions. Signals from outside of cells pass through receptors located on the cellular membrane and are transmitted to the nucleus of a cell through various biochemical reactions, where they express specific genes. This transfer of external signals into a cell is accomplished by protein interactions of several stages. For example, growth factors or cytokines bind to corresponding cell-surface receptors. This binding induces the receptors to cluster. The clustering of receptors by ligands induces clustering of the intracellular domains of the receptors, thereby causing interactions with signaling-related proteins. Through this signaling mechanism, intermediate proteins capable of transferring signals are produced by phosphorylation by protein kinases, dephosphorylation by protein phosphatases, or the like. As a result, the signals are transmitted to transcriptional activator proteins (Helden, C. H., (1995) *Cell* 80, 213-223). The activated transcriptional activators bind to DNAs and interact with basal transcriptional regulator proteins such as RNA polymerases to activate specific genes. Such interactions enable transcription to occur specifically in specific tissues during embryologic processes or in response to external stimulations. Abnormal modification, inhibition or acceleration of such interactions between specific proteins, which may be caused by intrusion of foreign matters, genetic modification of internal activator proteins, or the like, may be the cause of a disorder. Accordingly, there have been consistent researches because substances that can regulate the interactions may provide a way to treat the disorder.

The methods for analyzing the interactions of biomolecules, particularly the binding properties thereof, include traditional in vitro methods such as cross-linking, affinity chromatography, immunoprecipitation (IP), or the like. These

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methods require the production, isolation and purification of protein and are disadvantageous in that an information different from the actual interaction may be obtained depending on the buffer condition in the test tube, the secondary modification of extracted proteins, or the like.

In order to make up for these drawbacks of the in vitro methods, in-cell methods such as yeast two-hybrid (Y2H), fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (Bi-FC) techniques have been developed. These methods have advantages and disadvantages mentioned below.

Y2H is currently the most widely used technique along with immunoprecipitation. It is advantageous in that large-scale screening is possible using a gene library, but is disadvantageous in that investigation of membrane proteins or nuclear proteins such as transcriptase is difficult and there is a high probability of false positive. Besides, this method is inappropriate to find a substance capable of regulating protein-protein interactions. In the Y2H technique, the interaction between two proteins is detected based on the color change of colony to blue as X-gal is decomposed when β -galactosidase is expressed by the reporter gene. Since the screening technique of detecting the color change from blue back to white by a candidate substance is a negative screening, it is probable that a substance which has actually an inhibitory effect may be unnoticed. Further, since the detection itself is somewhat ambiguous, the technique is not suitable for general drug screening.

The FRET method provides good accuracy, but it is disadvantageous in that positioning of fluorescent proteins or fluorescent materials, which is required for the fluorescence resonance energy transfer to occur, is difficult, thereby having low rate of experimental success. The Bi-FC method is advantageous in that it is applicable to membrane proteins or nuclear proteins. However, like the FRET method, it is disadvantageous in that relative positioning of proteins for complementary binding is difficult, thereby having low rate of success.

Therefore, various modified methods have been proposed to overcome the disadvantages of the above-described methods. However, there is a consistent need for an effective method for detecting the binding of biomaterials. Particularly, a detecting system enabling the detection of proteins interacting with target proteins and enabling a more efficient detection of regulator materials that inhibit or promote the interactions between two proteins is urgently needed.

SUMMARY OF THE DISCLOSURE

The inventors of the present invention have researched to develop a method enabling the real-time detection of binding and interactions of materials in living cells. As a result, we have found that the interaction of a bait and a prey in a living cell can be detected in real time by using a first construct which includes a translocation module moving to a specific region in a cell in response to an external signal or via an internal signaling mechanism and a bait which is a target of an interaction, and a second construct which includes a prey which is another target of the interaction. The first construct and the second construct are labeled with a labeling material, so that the interaction of the bait and the prey in a living cell can be detected in real time by tracing their movements in the cell.

Accordingly, the present invention provides a novel method enabling the detection of bait and a prey which interact with each other.

To achieve the above object, the present invention provides the method for detecting interactions between bait and prey comprising the steps of:

(a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material; and

(b) detecting the distribution of the first construct and the second construct in the cell.

To achieve another object, the present invention provides the screening method for materials changing interaction between bait and prey comprising the steps of:

(a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material;

(b) allowing the bait of the first construct and the prey of the second construct to interact;

(c) treating with a signaling material to translocate the first construct to a plasma membrane of the cell; and

(d) detecting a translocation of the first construct and the second construct to a plasma membrane of the cell in the cell.

To achieve another object, the present invention provides the cells comprising (i) a first construct comprising bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material.

To achieve another object, the present invention provides the kit for detecting interactions between bait and prey comprising the said cells.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

The above and other aspects, features and advantages of the disclosed exemplary embodiments will be more apparent from the following detailed description taken in conjunction with the accompanying drawings in which:

FIG. 1 illustrates basic constructs and analysis indices of the present invention. 1) A first construct comprises a translocation module, a first labeling material and a bait. The first construct may comprise a single bait or a plurality of baits. 2) A second construct comprises a second labeling material and a prey. The second construct may comprise a single prey or a plurality of baits. 3) A signal S includes an extrinsic stimulation inducing the movement of the translocation module, intrinsic stimulation (DAG), and cellular concentration change of ATP or calcium intrinsically or extrinsically. 4) Interaction analysis indices include the distribution of the position of the first construct and second construct by the intrinsic or extrinsic characteristics of baits or preys, change of the distribution, translocation characteristics of the constructs, identification of complexes formed by one or more of the first and second constructs, or the like.

FIG. 2 illustrates a basic concept of binding and non-binding in accordance with the present invention. The bait and prey expressed in the cell are distributed randomly in the cytoplasm or nucleus. In response to a translocation signal, the first construct is carried toward the cell membrane by the translocation module (bait, red). The prey bound to the bait is also carried toward the cell membrane (A; prey, green). In contrast, the prey not bound to the bait does not change its

original distribution (B; prey, green). Accordingly, the movement of the prey toward the cell membrane reflects the binding between the bait and prey.

FIG. 3 schematically illustrates the analysis of binding and non-binding in accordance with the present invention. For example, if plasmid vectors wherein the first construct and the second construct are encoded, respectively, are introduced into a cell at the same time, the two constructs are overexpressed in the cell (see A), resulting in the binding of the bait and prey by the intrinsic interaction property. However, in this state, the binding cannot be recognized because the two fluorescent labels exist together as seen in FIG. 2 (see B). When an external stimulation (1 μ M PMA) is applied in order to confirm the interaction between baits and preys, the first construct comprising the translocation module moves toward the cell membrane, and the prey bound to the bait is also carried toward the cell membrane (see C). Accordingly, the binding between the bait and prey can be detected from the co-localized translocation of the two fluorescent labels. By labeling both the first construct and second construct with a labeling material and tracing them, the experimental accuracy can be improved as compared to when only the first construct or the second construct is labeled with a labeling material. If only the first construct is labeled with a labeling material, it cannot be confirmed whether the prey binds to the bait. And, if only the second construct is labeled with a labeling material, it cannot be confirmed whether the movement of the prey is due to the binding with the bait, in case the prey has mobility in response to an external stimulation or due to an internal cause.

FIG. 4 shows an example of first construct (A) and second construct (B) in accordance with the present invention. Basically, the first construct comprises a translocation module (TMD, TMA, TMB), a fluorescent protein such as red fluorescent protein (mR; mRFP) or green fluorescent protein (EGFP), and a multicloning gene sequence (Bait) for the bait. If it is needed to change the location of the bait or prey in the cell, the construct may further include a nuclear localization signal (NLS) or a nuclear export signal (NES). The second construct comprises a fluorescent protein (any fluorescent protein distinguishable from that of the first construct) for identifying the movement of the prey, and a multicloning gene sequence (Prey) for the prey. The fluorescent protein may be EGFP, mRFP, Azami Green (AzG), HCR, etc. A variety of distinguishable combinations may be attained using currently known fluorescent proteins, depending on the analysis tools (microscope, etc.) to be used.

FIG. 5 shows the change of the distribution of the first construct vector and second construct vector in response to an external stimulation. Basic first constructs with no bait cloned (i.e., without a bait) [TMD-EGFP-Bait (A, left panel), TMD-mRFP-Bait (A, right panel)] and basic second constructs with no prey [EGFP-Prey (B, left panel), mRFP-Prey (B, right panel)] were overexpressed in CHO-k1 cell line, and the distribution of the location of the two constructs was confirmed after applying an external stimulation (1 μ M PMA). The EGFP- or mRFP-labeled first construct moved toward the cell membrane in response to the external stimulation (see A), whereas the second construct not comprising the translocation module showed no change of fluorescence distribution in response to the external stimulation (see B). Accordingly, it can be seen that the change of the distribution of the second construct is a passive phenomenon occurring due to other factor (the first construct).

FIG. 6 shows the change of the distribution of the first construct (TMD-mRFP) depending on the PMA concentration. In order to optimize the translocation characteristic of

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the translocation module, the first construct vector (pTMD-mRFP-C3) shown in FIG. 5 was used to determine the optimum concentration of PMA (phorbol-12-myristate-13-acetate), which was used as an external stimulation. Detailed experimental procedures were the same as in FIG. 5. After treating with PMA at concentrations of 1 nM to 5 μ M, the cells in which red fluorescence moved toward the cell membrane were counted among randomly selected fluorescence-exhibiting cells. As seen in the table, IC₅₀ was measured as 35 nM. At 50 nM, 90% or more translocation was observed. At 100 nM or higher concentrations, movement of red fluorescence toward the cell membrane increased. Therefore, all experiments were carried out at 1 μ M, which is a concentration sufficient for the response and movement of most cells.

FIG. 7 compares translocation efficiency of the first constructs [TMD-mRFP (right panel), TMA-mRFP (left panel), TMB-mRFP (central panel)]. In order to determine the optimum translocation module for the first construct, translocation efficiency of the first construct vector shown in FIG. 5 (pTMD-mRFP-C3), a pTMA-mRFP-C3 vector comprising a TMD fragment (a vector prepared by inserting TMA translocation module into the mRFP-C3 vector, see Example 2), and a pTMB-mRFP-C3 vector (a vector prepared by inserting TMB translocation module into the mRFP-C3 vector, see Example 2) was evaluated. Detailed experimental procedures were the same as in FIG. 5. All the three constructs exhibited change of distribution in response to an external stimulation (see A). There was no statistical difference in the number of cells with changed distribution. However, when the intensity of fluorescence that moved toward the cell membrane was measured (see white lines in A and B), TMA and TMB showed comparable results, whereas TMD exhibited relatively distinct difference in the cytoplasm (denoted as C) and at the cell membrane (denoted as M). Also, fluorescence density at the cell membrane was higher than those of TMA and TMB. Therefore, all experiments were carried out using TMD which exhibited good translocation characteristic and translocation efficiency.

FIG. 8 shows the result of verifying the efficiency of NES sequence which limits the expression site of the first construct to the cytoplasm, in order to promote the efficacy of the present construct. In order to verify the effect of the NES sequence, a first construct (TMD-mRFP-Bait-NES) basic vector comprising the NES sequence (pTMD-mRFP-C3-NES vector) was prepared, and overexpressed in HEK-293 cell line along with a second construct (EGFP-Prey-NES) basic vector (pEGFP-C3-NES vector). The NES sequence-including first construct was observed mainly in the cytoplasm while the second construct was uniformly distributed in the whole cell (see A). After treating with PMA for 3 minutes, the first construct which had been uniformly distributed in the cytoplasm moved toward the cell membrane, whereas the second construct without including the NES sequence did not show any change in distribution (see B; left panel: first construct, central panel: second construct, right panel: merge).

FIG. 9 shows the result of verifying the efficiency of NLS sequence which limits the expression site of the first construct to the nucleus, in order to promote the efficacy of the present construct. In order to verify the effect of the NLS sequence, TMD-mRFP-Bait-NLS (pTMD-mRFP-C3-NLS vector) and EGFP-Prey-NLS (pEGFP-C3-NLS vector) basic vectors were prepared and overexpressed in HEK-293 cell line. The NLS sequence-including first construct and second construct were observed mainly in the nucleus (see A). After treating with PMA for 3 minutes, the first construct and second construct which had been uniformly distributed in the nucleus

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moved toward the nuclear membrane, whereas the second construct without the translocation module did not show any change in distribution (see B; left panel: first construct, central panel: second construct, right panel: merge).

FIG. 10 shows the result of analyzing the binding of p53 protein and SV40T protein using the method of the present invention. In order to verify the binding of the bait (p53 protein) and the prey (SV40T protein), TMD-mRFP-p53 (first construct) and EGFP-SV40T (second construct) were prepared and overexpressed in CHO-k1 cell line. After treating with PMA for 3 minutes, the two proteins which had been uniformly distributed in the cell (A) moved toward the cell membrane (B) (red fluorescence: translocation module+p53 protein, green fluorescence: SV40T protein). Accordingly, it was confirmed that the two proteins are bound to each other in the cell (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 11 shows the result of analyzing the binding of lysyl-tRNA synthetase (KRS) and JTV1 (p38), Gag and laminin receptor (LR). In order to verify the binding of p38, Gag and LR, which were expected to bind with the KRS protein, KRS (as second construct) and p38, Gag and LR (as first construct) were overexpressed in HEK-293 cell line. Fluorescence distribution in the cell before and after treatment with PMA was analyzed. (A) TMD-mRFP-p38 and AzG-KRS strongly moved toward the cell membrane in response to an external stimulation, whereas TMD-mRFP-Gag (B) or TMD-mRFP-LR (C) labeled with green fluorescence did not show translocation. Accordingly, it was confirmed that KRS binds with p38 among the three proteins (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 12 shows the result of analyzing the binding of RelA and I κ B in real time using a confocal laser fluorescence microscope. RelA and I κ B, which are known to form a complex in a cell, were prepared into TMD-mRFP-RelA and EGFP-I κ B, respectively, and overexpressed in CHO-k1 cell. Images were taken every 10 seconds, using a confocal laser fluorescence microscope. Prior to PMA treatment (0 min), fluorescence of the two proteins were uniformly distributed in the cytoplasm. About 10 seconds to 1 minute after the PMA treatment, the two fluorescences moved toward the cell membrane. Accordingly, it was confirmed that binding of two proteins in a living cell can be analyzed in real time. Hence, the present invention is useful for the study of cell signaling mechanisms in response to various stimulations including translocation signals (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 13 shows the result of analyzing the binding of three protein complexes. The two proteins (RelA and I κ B) of FIG. 12 are known to form an NF κ B-I κ B complex in a cell along with p50 protein. In order to verify whether the binding of a plurality of proteins can be analyzed, the second construct HcR-p50 was prepared by binding p50 protein with another fluorescent protein HcRed (HcR). The three constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescence signals (red, green and blue) reflecting the three proteins were uniformly distributed in the cytoplasm and nucleus prior to PMA treatment (A). However, after the PMA treatment (B), all of them moved toward the cell membrane. This result means that complexes composed of at least three proteins can be analyzed at the same time using the constructs of the present invention [from the left side, first panel: first construct (TMD-mRFP-RelA), second panel: second construct 1 (EGFP-I κ B), third panel: second construct 2 (HcR-p50), right panel: merge].

FIG. 14 shows the result of verifying the binding of positive candidates screened through Y2H. In order to reconfirm the binding of the candidates screened through Y2H, a screening method commonly used for screening of protein interaction, a first construct comprising OmpA protein as bait (TMD-mRFP-OmpA) and second constructs comprising EEF1A, FAM14B and DDX31 as prey candidates (EGFP-EEF1A, EGFP-FAM14B and EGFP-DDX31) were prepared. Combination of the bait and each of the prey candidates were over-expressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescences were uniformly distributed in the cell prior to PMA treatment. After the PMA treatment, the translocation modules binding with the OmpA protein moved toward the cell membrane (red). Of the green fluorescences reflecting the prey candidates that were expected to bind thereto, EEF1A (A) and FAM14B (B) remained in the cytoplasm and DDX31 (C) moved toward the cell membrane. Thus, it was confirmed that only one of the three candidates screened through Y2H participates in the binding. This result shows that the present invention can solve the false positive problem of Y2H (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 15 shows the result of analyzing the binding of p53 protein with mdm2 protein and the inhibition of binding by the anticancer drug nutlin3. In order to verify the effectiveness of the present invention in new drug development, experiments were carried out on the inhibition of binding of an anticancer drug. To this end, a first construct comprising p53 protein (TMD-mRFP-p53N) and a second construct comprising mdm2 protein (AzG-mdm2N) were prepared. The constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. First, in order to verify the binding of p53N and mdm2N, cells not treated with nutlin3 were compared before and after PMA treatment (A). The two proteins which had been uniformly distributed in the cytoplasm prior to the PMA treatment moved toward the cell membrane after the PMA treatment. This indicates that the two proteins are bound to each other. Next, in order to verify the binding inhibition effect of the anticancer drug nutlin3, cells were cultured in a medium containing 20 nM nutlin3 for 20 minutes. Then, fluorescence distribution was observed in real time before and after PMA treatment (B). In the nutlin3-pretreated cells, the first construct (red) comprising the translocation module and p53N moved toward the cell membrane, but the second construct (green) comprising only mdm2N and fluorescent protein with no translocation module did not show translocation toward the cell membrane. Thus, it was confirmed that the present invention is useful in verifying the inhibition of binding of p53 and mdm2 by the anticancer drug nutlin3. Therefore, the present invention can be used to screen inhibitors of binding of proteins or peptides (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 16 shows the result of analyzing the binding inhibition concentration of the anticancer drug nutlin3. In order to determine the optimum concentration of the anticancer drug nutlin3, the binding inhibition effect of which was confirmed in FIG. 15, the binding inhibition effect was analyzed at various concentrations. Nutlin3 was treated at 0, 0.5, 1, 5, 10, 25, 50, 100, 200 nM. The binding inhibition effect of nutlin3 was distinct from 5 nM. At 10 nM, the binding between the two proteins was inhibited in more than 50% of the cells. At 25 nM, binding was not observed in more than 90% of the cells. This result shows that the binding inhibition effect of a

binding inhibitor such as an anticancer drug can be precisely detected even at a very low concentration of 500 nM.

FIG. 17 shows the result of analyzing the binding of five protein complexes. In order to verify whether the binding of a plurality of proteins can be analyzed, the first construct TMD-HcR-p38 was prepared and each of second construct, BFP-KRS, CFP-RRS, GFP-DRS, YFP-p43 was prepared, respectively. The five constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescence signals (blue, cyan, green, yellow and red) reflecting the five proteins were uniformly distributed in the cytoplasm and nucleus prior to PMA treatment (A). However, after the PMA treatment (B), all of them moved toward the cell membrane. This result means that complexes composed of five proteins can be analyzed at the same time using the constructs of the present invention.

DETAILED DESCRIPTION OF THE DISCLOSURE

In the present invention, translocation of proteins in a cell in response to an external signal or via an internal signaling mechanism is monitored to detect the interaction between biomaterials in a cell directly and in real time. A first construct is designed by fusing a bait, which is a target of an interaction, with a translocation module, which relocates in response to an external signal or via an intrinsic signaling mechanism, and with a labeling material to trace it. Further, a second construct is designed to comprise a prey interacting with the bait and another labeling material enabling tracing thereof. The first construct and the second construct are made to exist in a cell at the same time. As a result, the interaction between them in a cell can be analyzed directly in real time.

Accordingly, the present invention provides the method for detecting interactions between bait and prey comprising the steps of:

(a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material; and

(b) detecting the distribution of the first construct and the second construct in the cell.

The present invention may provides the method for detecting interactions between bait and prey comprising the steps of:

(a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material;

(b) allowing the bait of the first construct and the prey of the second construct to interact; and

(c) detecting a translocation of the first construct and the second construct to a plasma membrane of the cell in the cell.

As used herein, the bait (i.e., the molecule of interest) and the prey (i.e., the target molecule) refer to the materials that are subject to an interaction. Each of the bait and the prey may be protein, polypeptide, small organic molecule, polysaccharide or polynucleotide, respectively. Preferably, they may be protein or polypeptide. Further, they may be a natural product, synthetic compound, chemical compound or a combination of two or more of them. For the purpose of detection or screening of an interaction, the bait may be a known material and the prey may be an unknown material. But, without being limited thereto, the bait and the prey may be interchangeably included in the first construct or second construct.

As used herein, a first labeling material and a second labeling material refer to a material capable of generating a signal that can be detected by those skilled in the art. Examples may include fluorescent materials, ligands, light-emitting materials, microparticles (or nanoparticles), redox molecules, radioactive isotopes, or the like. As for fluorescent materials, without being limited thereto, fluorescent protein, fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, fluoresceinisothiocyanate may be used. Among the above materials, as for fluorescent protein, those which are well known in the art may be used. Examples may include GFP (Green Fluorescent Protein); EGFP (Enhanced Green Fluorescent Protein); RFP (Red Fluorescent Protein); mRFP (Monomeric Red Fluorescent Protein); DsRed (*Discosoma* sp. red fluorescent protein); CFP (Cyan Fluorescent Protein); CGFP (Cyan Green Fluorescent Protein); YFP (Yellow Fluorescent Protein); AzG (Azami Green), HcR (HcRed, *Heteractis crispa* red fluorescent protein), BFP (Blue Fluorescent Protein). As for ligands, there is biotin derivative and as for luminescent, without being limited thereto, there are acridinium ester, luciferin, luciferase, or the like. As for microparticles (or nanoparticles), without being limited thereto, there are colloid gold, iron, colored latex and as for redox molecules, without being limited thereto, there are ferrocene, ruthenium complex compounds, bioluminescence, quinine, Ti ion, Cs ion, diimides, 1,4-benzoquinone, hydroquinone. As for radioactive isotopes, without being limited thereto, there are ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , ^{186}Re or the like. However, any one which could be used for detecting labeling materials can be used as well as the above exemplified materials.

Preferably, a first labeling material and a second labeling material of the present invention may be fluorescent proteins. More preferably, a first labeling material and a second labeling material of the present invention may be GFP; EGFP; RFP; mRFP; DsRed (*Discosoma* sp. red fluorescent protein); CFP (Cyan Fluorescent Protein); CGFP (Cyan Green Fluorescent Protein); YFP (Yellow Fluorescent Protein); AzG (Azami Green), HcR (HcRed, *Heteractis crispa* red fluorescent protein), or BFP (Blue Fluorescent Protein). At this time, it is preferred that a first labeling material and a second labeling material are different for distinction. More preferably, a first labeling material and a second labeling material of the present invention may have an amino acid sequence represented by SEQ ID NO:9 (EGFP), SEQ ID NO:11 (mRFP), SEQ ID NO:13 (AzG) or SEQ ID NO:15 (HcR) or a nucleotide sequence represented by SEQ ID NO:10 (EGFP), SEQ ID NO:12 (mRFP), SEQ ID NO:14 (AzG) or SEQ ID NO:16 (HcR).

In the present invention, the translocation module serves to move the first construct to a specific region in a cell. The translocation to the specific region may be induced by an external signal or induced intrinsically. The specific region in a cell refers to an intracellular structure which is separate, discreet and identifiable. Preferably, the specific region may be membranous (structures such as cell membrane, plasma membrane, nuclear membrane, etc.), organelles such as endoplasmic reticulum, Golgi apparatus, mitochondria, lysosome, etc., or other specific regions in a cell.

The translocation module may be different depending on the particular specific region in a cell. Preferably, it may be protein kinase C (PKC), including classical PKCs (cPKCs; PKC- α , PKC- β and PKC- γ), novel PKCs (nPKCs; PKC- δ , PKC- ϵ , PKC- ζ and PKC- θ), atypical PKCs (aPKCs; PKC- ξ and PKC- ι/κ) and their variants, known in the art. All of them commonly have the C1 domain. When diacylglycerol (DAG) or phorbol ester

(TPA or PMA) binds at the C1 domain, they are induced to move toward the cell membrane. Preferably, a variant of PKC may be used as the translocation module of the present invention. More preferably, the variant may be one from which the internal phosphorylation active site of PKC is removed in order to minimize interference caused by the internal signaling mechanism. More preferably, the translocation module of the present invention may have an amino acid sequence of SEQ ID NO: 1 (PRKCD), SEQ ID NO: 3 (TMA), SEQ ID NO: 5 (TMB) or SEQ ID NO: 7 (TMD), or a nucleotide sequence of SEQ ID NO: 2 (PRKCD), SEQ ID NO: 4 (TMA), SEQ ID NO: 6 (TMB) or SEQ ID NO: 8 (TMD).

From the analysis of translocation efficiency of several proteins including RasGRP, which migrates by signals, and C1 domains through preliminary experiments, it was confirmed that full-length PKCs (denoted as PRKCD) or mutants derived from PKC variants (denoted as TMD) of the present invention exhibited relatively superior translocation efficiency (see FIG. 7).

The first construct and second construct expressed may exist in cytosol region of cells. However, the first construct or second construct of the present invention may further include a nuclear localization signal (NLS) or nuclear export signal (NES). These may be further included to control the change of intracellular distribution depending on the intrinsic properties of the bait or prey, or on the particular cell line used in the experiment. In case an NLS is further added, it may direct the first construct and second construct to be distributed uniformly in the nucleus. And, if an NES is further added, it may direct the first construct and second construct to be distributed uniformly in the cytoplasm. Through this, it is possible to recognize whether the binding of the bait and the prey occurs in the cytoplasm or nucleus, or to induce the binding occur in the cytoplasm or nucleus. NLS may preferably have a sequence which is well known in the art (for example, SV40 T Antigen (PKKKRKV), Yeast histone H2B (GKKRSKV), Human c-myc (PAAKRVLKLD), Nucleoplasmin (KRPAATKKAGQAKKKKL), Human IL-5 (KKYTDGQKKKC-GEERRRVNQ), Human RB (KRSAGESSNPPLKLLR), Human p53 (KRALPNNTSSSPQPKKKP)) or the amino acid sequence represented by SEQ ID NO:17, more preferably it may have the amino acid sequence represented by SEQ ID NO:17 (GSGDEVEGVVEVAKKKSKKEKDK) or the nucleotide sequence represented by SEQ ID NO:18 which encode thereof (ggctctggtgatgaagtcgaaggagtggaagaagtagctaagaagaagagtaaaaggaaaggataaa). In addition, NES may have a sequence which is well known in the art (for example, Annexin II (VHEILCK-LSLE), mNet (TLWQF-LH-LLLD), hNet (TLWQF-LLQ-LLLD), MAPKK (ALQKK-LEE-LELD), PKI (ELALK-LAG-LDIN), Rev (LQLPLER-LTLD), Dsk-1 (SLEGAVSEIS-LR), Cyclin B1 (YLCQAFSDVI-LA), ANXII (STVHEILCK-LSLE), HIV-1 Rev (LQLPLER-LTLD), MEK-1 (ALQKK-LEE-LELD), PKI- α (ELALK-LAG-LDIN), I κ B- α (IQQLGQ-LTLE), RanBP1 (KBAEKLEA-LSVR), INI1 (DQRVIKLNH-VGNISLV)) or amino acid sequence represented by SEQ ID NO 19, more preferably it may have the amino acid sequence represented by SEQ ID NO:19 (DQRVIKLNH-VGNISLV) or the nucleotide sequence represented by SEQ ID NO:20 (gac-cagcgctcatcatcaagctgaacgcccattgtgggaacatttcctggtg) which encode thereof.

The detection of the distribution of the first construct and second construct in a cell may be carried out using a labeling material in accordance with a detection method commonly known in the art. For example, if the labeling material is a

fluorescent protein, a fluorescence microscope may be used to detect the distribution of the first construct and second construct in a cell.

Initially, both the first construct and second construct are randomly distributed in the cytoplasm or nucleus (a translocation module moving via an intrinsic signaling mechanism moves to a specific region. However, targeting to cytosol of each of first construct and second construct without translocation signal is preferred.). When receiving a translocation signal, the first construct is moved toward the cell membrane by the translocation module. At this time, the prey bound to the bait is also carried toward the cell membrane. In contrast, unless the prey is not bound to the bait, its distribution will not change. Accordingly, the binding of the bait and the prey can be recognized by the translocation of the prey toward the cell membrane (see FIGS. 2 and 3). Therefore, translocation of the second construct to the plasma membrane of the cell indicates interactions of the bait and the prey, since the second construct does not have intrinsic translocation module.

Thus, the method of the present invention enables real-time monitoring of direct binding or complex of biomolecules in a living cell through imaging, and provides the following advantages over existing techniques.

- 1) All bindings occurring in a living cell can be analyzed.
- 2) Analysis in tissue or individual level is possible.
- 3) Applicable to animal cells, yeast and bacteria.
- 4) No antibodies for the bait and prey are required.
- 5) Accurate analysis is possible because the positional change in a cell is monitored, differently from other methods where the whole cell is monitored.
- 6) 3-dimensional analysis is unnecessary because 2-dimensional positional change is analyzed.
- 7) It is not necessary to use the expensive confocal microscope because 3-dimensional analysis is unnecessary.
- 8) Binding analysis is possible for various cell organelles.
- 9) Not influenced by external environment as in the in vitro method, because binding occurring in a living cell is monitored.
- 10) The binding of a bait and a prey can be monitored in real time.
- 11) The binding of a bait with multiple preys can be monitored.
- 12) False positive can be minimized through all-or-none monitoring.
- 13) Interfering bindings due to inflow of extracellular material can be excluded ultimately by using only genes of a bait and a prey and stimulating materials.
- 14) Complementary screening of protein binding is possible through various modifications of the translocation module.
- 15) Detection of permanent binding, transient binding and instantaneously occurring interaction is possible.
- 16) Targets of currently developed signaling inhibitors can be specified.
- 17) Re-evaluation of inhibitors (drug repositioning/repurposing) is possible through target specification of inhibitor.
- 18) Screening of binding of a prey to an unknown biomaterial is possible using a mass marker library for the prey.
- 19) A high-throughput system can be implemented in association with a high-content screening (HCS) system.
- 20) Simple analysis is possible based on stimulation-free movement.
- 21) Binding properties can be analyzed for different signaling pathways by changing external stimulation.
- 22) Relative quantitation of the bait and prey is possible by labeling both the first construct and second construct with a labeling material.

23) False positive or false negative responses can be significantly reduced because the experimental errors related to the translocation of the prey in response to external stimulation or via intrinsic signaling mechanism can be simultaneously verified.

As described, the translocation module may move to a specific region in a cell in response to an external signal. Accordingly, the detection of an interaction between a bait and a prey in accordance with the present invention may further comprise treating with a signaling material. That is, the method may comprise:

- (a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material;
- (b) allowing the bait of the first construct and the prey of the second construct to interact;
- (c) treating with a signaling material to translocate the first construct to a plasma membrane of the cell; and
- (d) detecting a translocation of the first construct and the second construct to a plasma membrane of the cell in the cell.

The signaling material refers to a material which generates an external signal inducing the translocation of the translocation module. For example, if PKC is used as the translocation module, the signaling material may be phorbol-12-myristate 13-acetate (PMA; phorbol ester), 12-O-tetradecanoylphorbol-13-acetate (TPA), phorbol-12,13-dibutyrate (PDBu), adenosine triphosphate (ATP), tridecanoic acid, arachidonic acid, linoleic acid, DiC8, 130C937, PKC activation-related growth factors or other PKC activating materials.

PMA may be treated at a concentration of preferably 50 nM to 5 μ M, more preferably 1 μ M. If the PMA concentration is below 50 nM, translocation of the PKC translocation module may be insufficient. Otherwise, if it exceeds 5 μ M, excessive treatment of the chemical may result in undesired phenomena as cell death, signaling interference, or the like.

Also, the present invention provides the method for screening materials which alter interactions of bait and prey comprising the steps of:

- (a) preparing a cell including (i) a first construct including a bait, a first labeling material and a translocation module; and (ii) a second construct including a prey and a second labeling material;
- (b) treating with a test agent; and
- (c) detecting the distribution of the first construct and the second construct in the cell.

As used herein, the term "test agent" includes any substance, molecule, element, compound, entity or a combination thereof. For example, it includes protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, or the like, but is not limited thereto. Further, it may be a natural product, synthetic compound or chemical compound, or a combination of two or more of them. Unless specified otherwise, the terms agent, substance, material and compound may be used interchangeably.

The test agent screened or identified by the method of the present invention may comprise polypeptide, beta-turn mimetics, polysaccharide, phospholipid, hormone, prostaglandin, steroid, aromatic compound, heterocyclic compound, benzodiazepines, oligomeric N-substituted glycines, oligo-carbamates, saccharides, fatty acid, purine, pyrimidine or derivatives thereof, structural analogues or mixtures thereof. The said test agent may be derived from broad and various origins which comprise artificial synthesis or library of natural compounds. Preferably, the said test agent may be peptide of, for example, about 5-30, preferably 5-20, more preferably

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7-15 amino acids. The said peptide may be naturally generated protein, random peptide, or fragment of "biased" random peptide.

In addition, the said test agent may be "nucleic acid". The nucleic acid test agent may be naturally generated nucleic acid, random nucleic acid, or "biased" random nucleic acid. For example, the fragment of prokaryotic genome or eukaryotic genome may be used as described above.

Further, the test agent may be a small compound molecule (e.g., a molecule having a molecular weight of about 1,000 or smaller). Preferably, high throughput assay may be employed to screen a small molecular agent.

The change of the interaction between the bait and the prey may be either an inhibition or enhancement of the interaction. The inhibition of the interaction refers to the inhibition of the binding between the bait and the prey. In the method of the present invention, the inhibition of the interaction, for example, may be determined from the absence (or decrease of frequency, degree or extent) of translocation of the second construct when the test agent is treated, as compared to that comparable to the translocation of the first construct when the test agent is not treated. The enhancement of the interaction refers to the enhancement of the binding between the bait and the prey. In the method of the present invention, the enhancement of the interaction, for example, may be determined from the presence (or increase of frequency, degree or extent) of translocation of the second construct comparable to that of the first construct when the test agent is treated, as compared to the absence of translocation of the second construct when the test agent is not treated.

In addition, the said screening method may further comprise the step of treating with a signaling material. For example, it may be the method comprising the steps of:

(a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material and a translocation module; and (ii) a second construct comprising a prey and a second labeling material;

(b) allowing the bait of the first construct and the prey of the second construct to interact;

(c) treating with a test agent;

(d) treating with a signaling material to translocate the first construct to a plasma membrane of the cell; and

(e) detecting a translocation of the first construct and the second construct to a plasma membrane of the cell in the cell.

However, the step of treating with a signaling material does not need to be performed prior to the step of treating with a test reagent, and the skilled in the art may control the procedures.

In addition, the present invention provides a cell including (i) a first construct including a bait, a first labeling material and a translocation module; and (ii) a second construct including a prey and a second labeling material.

The cell may be a cell of an animal, plant, yeast or bacteria. Preferably, except for bacteria, it may be a cell capable of accepting the first construct introduced from outside well and having well-defined boundaries of cytoplasm, nucleus and organelles. More preferably, the cell may be CHO-k1 (ATCC CCL-61, *Cricetulus griseus*, hamster, Chinese), HEK293 (ATCC CRL-1573, *Homo sapiens*, human), HeLa (ATCC CCL-2, *Homo sapiens*, human), SH-SY5Y (ATCC CRL-2266, *Homo sapiens*, human), Swiss 3T3 (ATCC CCL-92, *Mus musculus*, mouse), 3T3-L1 (ATCC CL-173, *Mus musculus*, mouse), NIH/3T3 (ATCC CRL-1658, *Mus musculus*, mouse), L-929 (ATCC CCL-1, *Mus musculus*, mouse), Rat2 (ATCC CRL-1764, *Rattus norvegicus*, rat), RBL-2H3 (ATCC CRL-2256, *Rattus norvegicus*, rat), MDCK (ATCC CCL-34,

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Canis familiaris). In addition, the cell may be stem cells, cells extracted from tissues and the artificially made mimic cell membrane structure.

The present invention further provides a kit for detecting interaction comprising a cell comprising the first construct and the second construct of the present invention.

The kit of the present invention may further comprise a tool and/or reagent known in the art used for the detection of a labeling material, in addition to the cell comprising the first construct and second construct. The kit of the present invention may further include a tube, well plate, instruction manual, or the like, if necessary.

The experimental procedures, reagents and reaction conditions that can be used in the method of the present invention may be those commonly known in the art and will be readily understood by those skilled in the art.

In the present invention, the cell comprising the first construct and second construct may be prepared by a molecular biology technique known in the art. Although not limited thereto, expression vectors capable of expressing the first construct and the second construct, respectively, or an expression vector capable of expressing both the first construct and second construct may be introduced into a cell, so that the first construct and second construct are expressed by the expression vector(s). To this end, for the first construct, an expression vector comprising a promoter (first promoter) and a nucleotide encoding a bait, a first labeling material and a translocation module, which is operably linked thereto, may be constructed, and, for the second construct, an expression vector comprising a promoter (second promoter) and a nucleotide encoding a prey and a second labeling material, which is operably linked thereto, may be constructed. The two expression vectors may be simultaneously or sequentially introduced into a single cell, so that the first construct and second construct are expressed by the expression vectors. The sequence of the bait, the first labeling material and the translocation module in the nucleotide is not important, as long as the function of the present invention is exerted. The same is true of the nucleotide encoding the prey and the second labeling material.

The "promoter" means a DNA sequence regulating the expression of nucleic acid sequence operably linked to the promoter in a specific host cell, and the term "operably linked" means that one nucleic acid fragment is linked to other nucleic acid fragment so that the function or expression thereof is affected by the other nucleic acid fragment. Additionally, the promoter may include an operator sequence for controlling transcription, a sequence encoding a suitable mRNA ribosome-binding site, and sequences controlling the termination of transcription and translation. Additionally, it may be constitutive promoter which constitutively induces the expression of a target gene, or inducible promoter which induces the expression of a target gene at a specific site and a specific time, and examples thereof include a SV40 promoter, CMV promoter, CAG promoter (Hitoshi Niwa et al., *Gene*, 108:193-199, 1991; Monahan et al., *Gene Therapy*, 7:24-30, 2000), CaMV 35S promoter (Odell et al., *Nature* 313:810-812, 1985), Rsyn7 promoter (U.S. patent application Ser. No. 08/991,601), rice actin promoter (McElroy et al., *Plant Cell* 2:163-171, 1990), Ubiquitin promoter (Christensen et al., *Plant Mol. Biol.* 12:619-632, 1989), ALS promoter (U.S. patent application Ser. No. 08/409,297). Also usable promoters are disclosed in U.S. Pat. Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142, etc).

The introduction of expression vector to a cell may be performed by the transfection methods which are well known

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in the art, for example, calcium phosphate method, calcium chloride method, rubidium chloride method, microprojectile bombardment, electroporation, particle gun bombardment, Silicon carbide whiskers, sonication, PEG-mediated fusion, microinjection, liposome-mediated method, magnetic nanoparticle-mediated method.

Meanwhile, general recombinant DNA and molecular cloning techniques of the present invention are well known in the art and they are well describe in the following references (Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. (1989); by Silhavy, T. J., Bannan, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. (1984); and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987)).

Hereinafter, the embodiments of the present invention will be described in detail with reference to accompanying drawings.

FIG. 1 illustrates basic constructs and analysis indices of the present invention. 1) A first construct comprises a translocation module, a first labeling material and a bait. The first construct may comprise a single bait or a plurality of baits. 2) A second construct comprises a second labeling material and a prey. The second construct may comprise a single prey or a plurality of baits. 3) A signal S includes an extrinsic stimulation inducing the movement of the translocation module [growth factors, serum factors, PMA, etc. that induce the positional change of translocation module], intrinsic stimulation (DAG), and cellular concentration change of ATP or calcium intrinsically or extrinsically. 4) Interaction analysis indices include the distribution of the position of the first construct and second construct by the intrinsic or extrinsic characteristics of baits or preys, change of the distribution, translocation characteristics of the constructs, identification of complexes formed by one or more of the first and second constructs, or the like.

FIG. 2 illustrates a basic concept of binding and non-binding in accordance with the present invention. The bait and prey expressed in the cell are distributed randomly in the cytoplasm or nucleus. In response to a translocation signal, the first construct is carried toward the cell membrane by the translocation module (bait, red). The prey bound to the bait is also carried toward the cell membrane (A; prey, green). In contrast, the prey not bound to the bait does not change its original distribution (B; prey, green). Accordingly, the movement of the prey toward the cell membrane reflects the binding between the bait and prey.

FIG. 3 schematically illustrates the analysis of binding and non-binding in accordance with the present invention. For example, if plasmid vectors wherein the first construct and the second construct are encoded, respectively, are introduced into a cell at the same time, the two constructs are overexpressed in the cell (see A), resulting in the binding of the bait and prey by the intrinsic interaction property. However, in this state, the binding cannot be recognized because the two fluorescent labels exist together as seen in FIG. 2 (see B). When an external stimulation (1 μ M PMA) is applied in order to confirm the interaction between baits and preys, the first construct comprising the translocation module moves toward the cell membrane, and the prey bound to the bait is also carried toward the cell membrane (see C). Accordingly, the binding between the bait and prey can be detected from the co-localized translocation of the two fluorescent labels. By labeling both the first construct and second construct with a labeling material and tracing them, the experimental accuracy

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can be improved as compared to when only the first construct or the second construct is labeled with a labeling material. If only the first construct is labeled with a labeling material, it cannot be confirmed whether the prey binds to the bait. And, if only the second construct is labeled with a labeling material, it cannot be confirmed whether the movement of the prey is due to the binding with the bait, in case the prey has mobility in response to an external stimulation or due to an internal cause.

FIG. 4 shows an example of first construct (A) and second construct (B) in accordance with the present invention. Basically, the first construct comprises a translocation module (TMD, TMA, TMB), a fluorescent protein such as red fluorescent protein (mR; mRFP) or green fluorescent protein (EGFP), and a multicloning gene sequence (Bait) for the bait. If it is needed to change the location of the bait or prey in the cell, the construct may further include a nuclear localization signal (NLS) or a nuclear export signal (NES). The second construct comprises a fluorescent protein (any fluorescent protein distinguishable from that of the first construct) for identifying the movement of the prey, and a multicloning gene sequence (Prey) for the prey. The fluorescent protein may be EGFP, mRFP, Azami Green (AzG), HCR, etc. A variety of distinguishable combinations may be attained using currently known fluorescent proteins, depending on the analysis tools (microscope, etc.) to be used.

FIG. 5 shows the change of the distribution of the first construct vector and second construct vector in response to an external stimulation. Basic first constructs with no bait cloned (i.e., without a bait) [TMD-EGFP-Bait (A, left panel), TMD-mRFP-Bait (A, right panel)] and basic second constructs with no prey [EGFP-Prey (B, left panel), mRFP-Prey (B, right panel)] were overexpressed in CHO-k1 cell line, and the distribution of the location of the two constructs was confirmed after applying an external stimulation (1 μ M PMA). The EGFP- or mRFP-labeled first construct moved toward the cell membrane in response to the external stimulation (see A), whereas the second construct not comprising the translocation module showed no change of fluorescence distribution in response to the external stimulation (see B). Accordingly, it can be seen that the change of the distribution of the second construct is a passive phenomenon occurring due to other factor (the first construct).

FIG. 6 shows the change of the distribution of the first construct (TMD-mRFP) depending on the PMA concentration. In order to optimize the translocation characteristic of the translocation module, the first construct vector (pTMD-mRFP-C3) shown in FIG. 5 was used to determine the optimum concentration of PMA (phorbol-12-myristate-13-acetate), which was used as an external stimulation. Detailed experimental procedures were the same as in FIG. 5. After treating with PMA at concentrations of 1 nM to 5 μ M, the cells in which red fluorescence moved toward the cell membrane were counted among randomly selected fluorescence-exhibiting cells. As seen in the table, IC₅₀ was measured as 35 nM. At 50 nM, 90% or more translocation was observed. At 100 nM or higher concentrations, movement of red fluorescence toward the cell membrane increased. Therefore, all experiments were carried out at 1 μ M, which is a concentration sufficient for the response and movement of most cells.

FIG. 7 compares translocation efficiency of the first constructs [TMD-mRFP (right panel), TMA-mRFP (left panel), TMB-mRFP (central panel)]. In order to determine the optimum translocation module for the first construct, translocation efficiency of the first construct vector shown in FIG. 5 (pTMD-mRFP-C3), a pTMA-mRFP-C3 vector comprising a TMD fragment (a vector prepared by inserting TMA translo-

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cation module into the mRFP-C3 vector, see Example 2), and a pTMB-mRFP-C3 vector (a vector prepared by inserting TMB translocation module into the mRFP-C3 vector, see Example 2) was evaluated. Detailed experimental procedures were the same as in FIG. 5. All the three constructs exhibited change of distribution in response to an external stimulation (see A). There was no statistical difference in the number of cells with changed distribution. However, when the intensity of fluorescence that moved toward the cell membrane was measured (see white lines in A and B), TMA and TMB showed comparable results, whereas TMD exhibited relatively distinct difference in the cytoplasm (denoted as C) and at the cell membrane (denoted as M). Also, fluorescence density at the cell membrane was higher than those of TMA and TMB. Therefore, all experiments were carried out using TMD which exhibited good translocation characteristic and translocation efficiency.

FIG. 8 shows the result of verifying the efficiency of NES sequence which limits the expression site of the first construct to the cytoplasm, in order to promote the efficacy of the present construct. In order to verify the effect of the NES sequence, a first construct (TMD-mRFP-Bait-NES) basic vector comprising the NES sequence (pTMD-mRFP-C3-NES vector) was prepared, and overexpressed in HEK-293 cell line along with a second construct (EGFP-Prey-NES) basic vector (pEGFP-C3-NES vector). The NES sequence-including first construct was observed mainly in the cytoplasm while the second construct was uniformly distributed in the whole cell (see A). After treating with PMA for 3 minutes, the first construct which had been uniformly distributed in the cytoplasm moved toward the cell membrane, whereas the second construct without including the NES sequence did not show any change in distribution (see B; left panel: first construct, central panel: second construct, right panel: merge).

FIG. 9 shows the result of verifying the efficiency of NLS sequence which limits the expression site of the first construct to the nucleus, in order to promote the efficacy of the present construct. In order to verify the effect of the NLS sequence, TMD-mRFP-Bait-NLS (pTMD-mRFP-C3-NLS vector) and EGFP-Prey-NLS (pEGFP-C3-NLS vector) basic vectors were prepared and overexpressed in HEK-293 cell line. The NLS sequence-including first construct and second construct were observed mainly in the nucleus (see A). After treating with PMA for 3 minutes, the first construct and second construct which had been uniformly distributed in the nucleus moved toward the nuclear membrane, whereas the second construct without the translocation module did not show any change in distribution (see B; left panel: first construct, central panel: second construct, right panel: merge).

FIG. 10 shows the result of analyzing the binding of p53 protein and SV40T protein using the method of the present invention. In order to verify the binding of the bait (p53 protein) and the prey (SV40T protein), TMD-mRFP-p53 (first construct) and EGFP-SV40T (second construct) were prepared and overexpressed in CHO-k1 cell line. After treating with PMA for 3 minutes, the two proteins which had been uniformly distributed in the cell (A) moved toward the cell membrane (B) (red fluorescence: translocation module+p53 protein, green fluorescence: SV40T protein). Accordingly, it was confirmed that the two proteins are bound to each other in the cell (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 11 shows the result of analyzing the binding of lysyl-tRNA synthetase (KRS) and JTV1 (p38), Gag and laminin receptor (LR). In order to verify the binding of p38, Gag and LR, which were expected to bind with the KRS protein, KRS

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(as second construct) and p38, Gag and LR (as first construct) were overexpressed in HEK-293 cell line. Fluorescence distribution in the cell before and after treatment with PMA was analyzed. (A) TMD-mRFP-p38 and AzG-KRS strongly moved toward the cell membrane in response to an external stimulation, whereas TMD-mRFP-Gag (B) or TMD-mRFP-LR (C) labeled with green fluorescence did not show translocation. Accordingly, it was confirmed that KRS binds with p38 among the three proteins (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 12 shows the result of analyzing the binding of RelA and IκB in real time using a confocal laser fluorescence microscope. RelA and IκB, which are known to form a complex in a cell, were prepared into TMD-mRFP-RelA and EGFP-IκB, respectively, and overexpressed in CHO-k1 cell. Images were taken every 10 seconds, using a confocal laser fluorescence microscope. Prior to PMA treatment (0 min), fluorescences of the two proteins were uniformly distributed in the cytoplasm. About 10 seconds to 1 minute after the PMA treatment, the two fluorescences moved toward the cell membrane. Accordingly, it was confirmed that binding of two proteins in a living cell can be analyzed in real time. Hence, the present invention is useful for the study of cell signaling mechanisms in response to various stimulations including translocation signals (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 13 shows the result of analyzing the binding of three protein complexes. The two proteins (RelA and IκB) of FIG. 12 are known to form an NFκB-IκB complex in a cell along with p50 protein. In order to verify whether the binding of a plurality of proteins can be analyzed, the second construct HcR-p50 was prepared by binding p50 protein with another fluorescent protein HcRed (HcR). The three constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescence signals (red, green and blue) reflecting the three proteins were uniformly distributed in the cytoplasm and nucleus prior to PMA treatment (A). However, after the PMA treatment (B), all of them moved toward the cell membrane. This result means that complexes composed of at least three proteins can be analyzed at the same time using the constructs of the present invention [from the left side, first panel: first construct (TMD-mRFP-RelA), second panel: second construct 1 (EGFP-IκB), third panel: second construct 2 (HcR-p50), right panel: merge].

FIG. 14 shows the result of verifying the binding of positive candidates screened through Y2H. In order to reconfirm the binding of the candidates screened through Y2H, a screening method commonly used for screening of protein interaction, a first construct comprising OmpA protein as bait (TMD-mRFP-OmpA) and second constructs comprising EEF1A, FAM14B and DDX31 as prey candidates (EGFP-EEF1A, EGFP-FAM14B and EGFP-DDX31) were prepared. Combination of the bait and each of the prey candidates were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescences were uniformly distributed in the cell prior to PMA treatment. After the PMA treatment, the translocation modules binding with the OmpA protein moved toward the cell membrane (red). Of the green fluorescences reflecting the prey candidates that were expected to bind thereto, EEF1A (A) and FAM14B (B) remained in the cytoplasm and DDX31 (C) moved toward the cell membrane. Thus, it was confirmed that only one of the three candidates screened through Y2H participates in the binding. This result shows that the present

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invention can solve the false positive problem of Y2H (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 15 shows the result of analyzing the binding of p53 protein with mdm2 protein and the inhibition of binding by the anticancer drug nutlin3. In order to verify the effectiveness of the present invention in new drug development, experiments were carried out on the inhibition of binding of an anticancer drug. To this end, a first construct comprising p53 protein (TMD-mRFP-p53N) and a second construct comprising mdm2 protein (AzG-mdm2N) were prepared. The constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. First, in order to verify the binding of p53N and mdm2N, cells not treated with nutlin3 were compared before and after PMA treatment (A). The two proteins which had been uniformly distributed in the cytoplasm prior to the PMA treatment moved toward the cell membrane after the PMA treatment. This indicates that the two proteins are bound to each other. Next, in order to verify the binding inhibition effect of the anticancer drug nutlin3, cells were cultured in a medium containing 20 nM nutlin3 for 20 minutes. Then, fluorescence distribution was observed in real time before and after PMA treatment (B). In the nutlin3-pretreated cells, the first construct (red) comprising the translocation module and p53N moved toward the cell membrane, but the second construct (green) comprising only mdm2N and fluorescent protein with no translocation module did not show translocation toward the cell membrane. Thus, it was confirmed that the present invention is useful in verifying the inhibition of binding of p53 and mdm2 by the anticancer drug nutlin3. Therefore, the present invention can be used to screen inhibitors of binding of proteins or peptides (left panel: first construct, central panel: second construct, right panel: merge).

FIG. 16 shows the result of analyzing the binding inhibition concentration of the anticancer drug nutlin3. In order to determine the optimum concentration of the anticancer drug nutlin3, the binding inhibition effect of which was confirmed in FIG. 15, the binding inhibition effect was analyzed at various concentrations. Nutlin3 was treated at 0, 0.5, 1, 5, 10, 25, 50, 100, 200 nM. The binding inhibition effect of nutlin3 was distinct from 5 nM. At 10 nM, the binding between the two proteins was inhibited in more than 50% of the cells. At 25 nM, binding was not observed in more than 90% of the cells. This result shows that the binding inhibition effect of a binding inhibitor such as an anticancer drug can be precisely detected even at a very low concentration of 500 nM.

FIG. 17 shows the result of analyzing the binding of five protein complexes. In order to verify whether the binding of a plurality of proteins can be analyzed, the first construct TMD-HcR-p38 was prepared and each of second construct, BFP-KRS, CFP-RRS, GFP-DRS, YFP-p43 was prepared, respectively. The five constructs were overexpressed in HEK-293 cell line and their binding was observed using a confocal laser fluorescence microscope. Fluorescence signals (blue, cyan, green, yellow and red) reflecting the five proteins were uniformly distributed in the cytoplasm and nucleus prior to PMA treatment (A). However, after the PMA treatment (B), all of them moved toward the cell membrane. This result means that complexes composed of five proteins can be analyzed at the same time using the constructs of the present invention.

Accordingly, the present invention provides a method capable of detecting bindings and interactions occurring in a living cell in real time, and a method for screening a material that alters the interaction. The method of the present invention

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overcomes the disadvantages including inaccuracy and complexity of existing biomaterial interaction detection techniques, including in vitro method (in vitro and biochemical techniques), antibody binding techniques (antibody precipitation), fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (Bi-FC) and fluorescence correlation spectroscopy (FCS) techniques, etc. By labeling both constructs to promote accuracy, the present invention provides a novel real-time, antibody-free analysis.

EXAMPLES

Hereinafter, the present invention will be described in detail referring to the examples.

However, the following examples are for illustrative purposes only and are not intended to limit the scope of the present invention.

Example 1

Animal Cell Line and Transformation

<1-1> Animal Cell Line and Culturing

CHO-k1 (ATCC CCL-61, *Cricetulus griseus*, hamster, Chinese), HEK293 (ATCC CRL-1573, *Homo sapiens*, human), HeLa (ATCC CCL-2, *Homo sapiens*, human) and SH-SY5Y (ATCC CRL-2266, *Homo sapiens*, human) cell lines were used. The animal cells were cultured according to the instructions of ATCC (American Type Culture Collection) for the individual cells. CHO-k1 cells were cultured using F-12 medium, and HEK293, HeLa and SH-SY5Y cells were cultured using DMEM medium. Other culturing condition was the same. The cells were cultured as follows (Those skilled in the art may modify the specific conditions depending on purposes.). The cells were cultured in pH 7.4 medium (F-12 and DMEM) containing 25 mM HEPES, 10% fetal bovine serum (FBS, v/v), 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂ incubator maintained at 37° C.

<1-2> Transformation of Cell Line

In the Examples of the present invention, genes were introduced into the cells using ExGene 500 (Fermentas Life Science), one of the liposome-based techniques. All the conditions for the gene introduction including gene concentration were pursuant to the manufacturer's instructions. More specifically, after transferring the subcultured cells to a 12-well plate with a cover slip, followed by culturing for a day, the culture medium was replaced with 0.9 ml of fresh medium. About 1 µg of the transformation sample was added to 0.1 ml of 150 mM NaCl solution. After completely mixing, 3.3 µl of ExGene reagent was added and mixed by vortexing for 15 seconds. The resultant solution was allowed to stand at room temperature for 10 minutes and then added to each well of the 12-well plate in which the cells were growing. The cells were allowed to be transformed by culturing for 18 hours.

Example 2

Design and Preparation of First Construct and Second Construct

<2-1> Design and Preparation of First Construct

The first construct may be fused construct comprising a translocation module capable of moving the protein uniformly expressed in the cytoplasm toward the cell membrane, a fluorescent protein analyzable using a microscope, and a bait.

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Vectors expressing the first construct (see A of FIG. 4) were prepared as follows. Translocation modules were prepared by PCR using the following templates and primers and inserted at the *NheI*/*AgeI* site of a pEGFP-C3 vector (GenBank Accession No. U57607; Clontech Catalog No. #6082-1, SEQ ID NO: 21) and a pmRFP-C3 vector (mRFP; GenBank Accession No. DQ903889, SEQ ID NO: 22), thereby completing the vectors.

The TMD translocation module was prepared by PCR using a pCMV-SPORT6-PRKCD vector [GenBank Accession No. BC043350; purchased from Open Biosystems (see the world wide web at (www.openbiosystems.com); Catalog No. EHS1001-410108-BC043350] as template and SEQ ID NO: 23 (PRKCD-F; 5'-GAAGCTAGCCGCCACCATG-GCGCCGTTCTGC-3') and SEQ ID NO: 24 (PRKCD-R; 5'-GAAACCGGTGGATCTTCCAGGAGGT-GCTCGAATTTGG-3') as primers. And, the TMA translocation module was prepared by PCR using a pCMV-SPORT6-PRKCD vector as template and SEQ ID NO: 25 (TMA-F; 5'-GAAGCTAGCCGCCACCATGAAACAGGC-CAAAATCCACTACATC-3') and SEQ ID NO: 26 (TMA-R; 5'-GAAACCGGTGGAGTGTCCCGGCTGTTGGCCGC-3') as primers. Further, the TMB translocation module was prepared by PCR using a pCMV-SPORT6-PRKCD vector as template and SEQ ID NO: 27 (TMB-F; 5'-GCAGCTAGC-CGCCACCATGCAGAAAGAACGCTTCAACATCG-3') and SEQ ID NO: 28 (TMB-R; 5'-GCAACCGGTGGGGC-CTCAGCCAAAAGCTTCTG-3') as primers. Further, the TMB translocation module was prepared by PCR using a pCMV-SPORT6-PRKCD vector as template and SEQ ID NO: 27 (TMB-F; 5'-GCAGCTAGCCGCCACCATGCA-GAAAGAACGCTTCAACATCG-3') and SEQ ID NO: 28 (TMB-R; 5'-GCAACCGGTGGGGCCTCAGC-CAAAAGCTTCTG-3') as primers.

<2-2> Design and Preparation of Mutated First Construct

Since the translocation module included in the first construct is derived from a protein kinase, it intrinsically has the function of phosphorylation. There is a risk that cell signaling and protein interaction in the cells where the constructs are overexpressed may be inhibited or interfered by the intrinsic function of phosphorylation. Accordingly, in the present invention, mutation is induced to deprive the phosphorylation of the translocation module. In order to substitute the 311 st amino acid (tyrosine) and the 378th amino acid (lysine) of the kinase used in the present invention, which are known as very important phosphorylation sites, with phenylalanine (Y313F) and arginine, respectively, mutagenesis by PCR was performed and, finally, TMD translocation module was prepared.

To this end, genes were amplified using a forward primer (PKCD-F; 5'-GAAGCTAGCCGCCACCATGGCGCCGTTCTGC-3', SEQ ID NO: 29) and a modified reverse primer (Y313F-R; 5'-GAAACCTGAAATATCCCAAC-3', SEQ ID NO: 30) under a first PCR condition, and then genes were amplified using a modified forward primer (Y313F-F; 5'-GT-TGGGATATTTCAGGGTTTC-3', SEQ ID NO: 31) and a reverse primer (PKCD-R; 5'-GAAACCGGTGGATCTTC-CAGGAGGTGTCGAATTTGG-3', SEQ ID NO: 32) under a second PCR condition. The PCR product obtained from the first and second PCR were used as template for a third PCR using forward and reverse primers. The resultant gene is a Y313F mutant wherein the nucleotide sequence TAT is substituted by TTT. Using the gene as template, a K378R mutant wherein AAG is substituted by AGG was prepared through the same experimental procedure, using a forward primer (SEQ ID NO: 29), a reverse primer (SEQ ID NO: 32), a modified forward primer (K378R-F: 5'-TTTGCCATCA

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GGGCCCTCAAG-3', SEQ ID NO: 33) and a modified reverse primer (K378R-R: 5'-CTTGAGGGCCCTGATGGCAAA-3', SEQ ID NO: 34).

<2-3> Adhesion of NLS and NES to First Construct

Since the proteins existing in the organisms have their own distribution characteristics (targeting), the bait and prey may have targeting sites in the cell organelles. In this case, the detection of the change of the location of the first construct and second construct may be difficult. Accordingly, there is a need to control the distribution to the cytoplasm or nucleus so that the binding between the bait and prey can be verified through experiments. To this end, a vector capable of controlling the locational distribution of the bait and prey was prepared using a nuclear exclusion signal (NES) which targets a protein to the cytoplasm and a nuclear localization signal (NLS) which targets a protein to the nucleus.

The vector for locational control makes it possible to recognize whether the binding between the bait and prey occurs in the cytoplasm or in the nucleus. That is, assuming that the bait and prey bind in the cytoplasm and move toward cytoplasm, a combination of a first construct and a second construct both containing an NES allows the second construct to bind with the first construct and to move toward the cell membrane by PMA treatment, whereas a combination of constructs both containing an NLS allows only the first construct to move toward the nuclear membrane, not showing the movement of the second construct. In contrast, assuming that the bait and prey bind only in the nucleus, a combination of constructs both containing an NLS allows the constructs to move toward the nuclear membrane by PMA treatment, whereas a combination of constructs both containing an NES allows only the first construct to move toward the cell membrane, not showing the movement of the second construct.

The first construct and second construct comprising an NLS were prepared through a series of conventional PCR cloning method using the following primers. A first PCR was carried out using a pmRFP-C3 vector as template and using SEQ ID NO: 35 (NLS-F-1: 5'-AGTAAAAAGGAAAAG-GATAAATAGATAACTGATCATAATCAGCC-3') and SEQ ID NO: 36 (NLS-R: 5'-GCTGCAATAAACAAGTTAA-CAAC-3') as primers. A second PCR was carried out using the resultant PCR product as template and using SEQ ID NO: 37 (NLS-F-2: 5'-TGGAAGAAGTAGCTAAGAAGAA-GAGTAAAAAGGAAAAGGATAAA-3') and SEQ ID NO: 36 (NLS-R) as primers. Similarly, a third PCR was carried out using SEQ ID NO: 38 (NLS-F-3: 5'-TCCGGTGAT-GAAGTGAAGGAGTGAAGAAG-TAGCTAAGAAGAA-3') and SEQ ID NO: 36 (NLS-R) as primers, and a fourth PCR was carried out using SEQ ID NO: 39 (NLS-F-4: 5'-GCTGGATCCAGGCTCTGGTGAT-GAAGTGAAGG-3') and SEQ ID NO: 36 (NLS-R). Thus obtained gene fragments were inserted at the *Bam*HI/*Hpa*I site of the first construct vector or the second construct vector.

The first construct and second construct comprising an NES were prepared in the same manner, using the following primers. A first PCR was carried out using a pmRFP-C3 vector as template and using SEQ ID NO: 40 (NES-F-1: 5'-GTGGGAAACATTTCCCTGGTGTA-GATAACTGATCATAATCAGCC-3') and SEQ ID NO: 41 (NES-R: 5'-GCTGCAATAAACAAGTTAACAAC-3') as primers. A second PCR was carried out using the resultant PCR product as template and using SEQ ID NO: 42 (NES-F-2: 5'-GTCATCATCAAGCTGAACGCCCATGTGG-GAAACATTTCCCTGGT-3') and SEQ ID NO: 41 (NES-R) as primers. Similarly, a third PCR was carried out using SEQ ID NO: 43 (NES-F-3: 5'-GTCGGATCCAGAC-CAGCGGTCATCATCAAGCTGAACGCC-3') and SEQ

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ID NO: 41 (NES-R) as primers. Thus obtained gene fragments were inserted at the BamHI/HpaI site of the first construct vector or the second construct vector.

<2-4> Design and Preparation of Second Construct

The second construct comprises a labeling material for analyzing the movement of the prey which has characteristic for binding with the bait of the first construct. The second construct was prepared using a fluorescent material other than used in the first construct. Using green fluorescent protein (EGFP, AzG), red fluorescent protein (mRFP) and infrared fluorescent protein (HcR), the second construct was prepared by the method described for the first construct.

When EGFP was used as the second labeling material, a pEGFP-C3 vector (Clontech) was used. When mRFP was used, a pmRFP-C3 vector was used. When AzG was used, a pAzG-C3 vector was used. And, when HcR was used, a pHcR-C3 vector was used. The C3 vectors had been prepared by substituting the EGFP gene sequence site of the pEGFP-C3 vector with AzG and HcR genes, as follows.

The pAzG-C3 vector was prepared as follows. PCR was carried out using a pPM-mAG1 vector (purchased from MBL, Catalog No. AM-V0203; Karasawa, S., et al. 2003, *J. Biol. Chem.* 278, 34167-34171) as template and using SEQ ID NO: 44 (AzG-F: 5'-GGCACCGGTCGCCACCATG-GACCCCATGGTGAGTGTGAT-3') and SEQ ID NO: 45 (AzG-R: 5'-GGCAGATCTGACAGCTTGGCCTGACTCG-GCAGCAT-3') as primers. Then, the EGFP nucleotide sequence of the pEGFP-C3 vector was substituted at the AgeI/NotI site by the resultant PCR product.

The pHcR-C3 vector was prepared as follows. PCR was carried out using pHcRed-Tandem-N1 (purchased from Avrogen, Catalog No. FP204; Gurskaya et al., 2001, *FEBS Lett.* 507, 16-20.) as template and using SEQ ID NO: 46 (HcR-F: 5'-GCCACCGGTCGCCACCATGGTGAG-3') and SEQ ID NO: 47 (HcR-R: 5'-GCCGCGGCCGCTTATCAGT-TGGCCTTCTCGGGCAGGTC-3') as primers. Then, the EGFP nucleotide sequence of the pEGFP-C3 vector was substituted at the AgeI/NotI site by the resultant PCR product.

Example 3

Verification of Translocation Characteristics of First Construct and Second Construct

<3-1> Verification of Expression of Constructs and Analysis of Translocation Characteristics

A cover slip containing the cells in which the first construct and second construct vectors had been introduced was fixed to a perfusion chamber and mounted on the object stage of a confocal laser fluorescence microscope (Carl Zeiss LSM510). Images of the construct vectors were taken before and after external stimulation (treatment with 1 μ M PMA).

488 nm argon laser (EGFP or AzG), 543 nm HeNe laser (mRFP) or 561 nm DPSS laser (HcR) of the confocal laser fluorescence microscope was used to excite the fluorescent label, and the fluorescence signal generated by each fluorescent label was filtered through the band path filter BP505-530 (EGFP or AzG), long path filter LP560 or BP560-630 (mRFP) or long path filter LP650 (HcR). Images were taken after completely removing the interference between the fluorescence.

As a result, the green or red fluorescence emitted by the first construct vector comprising the translocation module (TMD) moved toward the cell membrane (see FIG. 5A), whereas the green or red fluorescence emitted by the second construct vector with no translocation module was uniformly distributed in the cytoplasm as before the stimulation (see FIG. 5B).

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Accordingly, it can be seen that the second construct vector does not respond to the external stimulation and that the movement of the second construct toward the cell membrane necessarily requires the binding of the bait and prey.

<3-2> Verification of Translocation of First Construct and Optimum Concentration of External Stimulant

Translocation efficiency of the first construct using the pTMD-mRFP-C3 vector depending on the concentration of external stimulant PMA was measured as follows. The expression vector of the first construct was transformed into CHO-k1 cells and overexpressed. After 18 hours of culturing, PMA was treated at 1, 5, 10, 20, 40, 50, 80 and 100 nM and 1 and 5 μ M. 5 minutes after the PMA treatment, the cells were fixed with 3.8% formaldehyde and observed using a confocal microscope (Carl Zeiss LSM 510). 200 cells exhibiting red fluorescence were randomly selected and the distribution of the red fluorescence at the cell membrane was measured for each concentration.

As a result, as seen in FIG. 6, translocation was observed in 90% or more of the cells at 50 nM. At 100 nM or above, translocation of the red fluorescence toward the cell membrane increased. Therefore, it can be seen that treatment at a concentration of 50 nM to 5 μ M is preferred, but treatment at 1 μ M, which is a concentration sufficient for the response and movement of most cells, is effective.

Example 4

Analysis of Binding in Cell in Real Time Using First Construct and Second Construct

<4-1> Analysis of Binding Between p53 Protein and SV40T Antigen in Cell

For the binding between p53, which is known as a potential carcinogenic gene, and SV40T antigen, which is known to bind to p53, there is a commercialized binding screening system based on IP. Analysis was carried out using the same proteins in order to verify the basic binding detection ability of the present invention. In order to verify the binding between p53 and SV40T antigen in cell, a first construct in which the p53 protein is fused (TMD-mRFP-p53) and a second construct in which the SV40T antigen is fused were prepared as follows.

The first construct TMD-mRFP-p53 was prepared as follows. PCR was carried out using a pGBK-p53-GAL4 vector (p53; GenBank Accession No. AF161020) as template and using SEQ ID NO: 48 (p53-F: 5'-GAAGAATTCTGATGCTGTACACCGAGACCCCTGGG-3') and SEQ ID NO: 49 (p53-R: 5'-GAAGGATCCCCGTCAGTCTGAGTCAGGCC-CCCACTT-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pTMD-mRFP-C3 vector (a vector obtained by inserting a TMD sequence into an mRFP-C vector, see Example 2).

The second construct EGFP-SV40T was prepared as follows. PCR was carried out using a pGADT7-SV40T-GAL4 vector (SV40T; GenBank Accession No. BC014270) as template and using SEQ ID NO: 50 (SV40T-F: 5'-GAAGAATTCTGATGGGAAGTATGAATGGGAGCAG-3') and SEQ ID NO: 51 (SV40T-R: 5'-GAAGGATCCCCGTTATGTTTCAGGTTTCAGGGGG-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pEGFP-C3 vector.

The first construct (TMD-mRFP-p53) and the second construct (EGFP-SV40T) were introduced into CHO-k1 cells according to the procedure described in Example <1-2> (Ex-Gene 500) and allowed to be expressed. After 18 hours of culturing, followed by treatment with 1 μ M PMA for 5 min-

utes, fluorescence distribution of the two constructs was observed as in Example <3-1>.

As seen in FIG. 10, both the red fluorescence (left panel) emitted by the bait p53 protein bound to the translocation module and the green fluorescence (central panel) emitted by the prey SV40T moved toward the cell membrane after the PMA treatment. In contrast, considering that translocation toward the cell membrane did not occur in a control group wherein only the second construct not bound to SV40T was expressed (result not shown), it can be seen that the prey SV40T binds with the bait p53 in the cell. Also, because the change of distribution of prey was appeared due to the change of distribution of bait, to which a translocation module was attached, it is confirmed that the two proteins bind to each other in cell.

<4-2> Analysis of Binding Between KRS Protein and p38 (AIMP2) Protein

Lysyl-tRNA synthetase (KRS), known as a multifunctional pathogenic gene, is known to form a complex with p38/AIMP2 protein and at least 3 other aminoacyl-tRNA synthetases (ARSB) (*J. Cell Science*, 2004, 117, 3725-3734, references therein). Accordingly, in order to verify the possibility of analyzing the binding of KRS protein and p38 protein in a cell in real time, the applicability in various animal cells, the diversity of fluorescent labels, and the exchangeability of the bait and prey, analysis was carried out using p38, which is known to bind with KRS, and using Gag and LR, which are expected as potential binding proteins.

To this end, a first construct (TMD-mRFP-p38, TMD-mRFP-Gag or TMD-mRFP-LR) and a second construct (AzG-KRS) were prepared as follows. AzG was used instead of EGFP as the fluorescence for the second construct.

The first construct TMD-mRFP-p38 was prepared as follows. PCR was carried out using a pGEX-4T1-p38 vector (p38; GenBank Accession No. NM_006303) as template and using SEQ ID NO: 52 (p38-F: 5'-GTCTCGAGATGCGATGTACCAGGTAAAG-3') and SEQ ID NO: 53 (p38-R: 5'-GTCGGATCCTTAAAAAGGAGCCAGGTTTTC-3') as primers. The resultant PCR product was inserted at the XhoI/BamHI site of the pTMD-mRFP-C3 vector.

TMD-mRFP-Gag was prepared as follows. PCR was carried out using a pGEX-4T1-Gag vector (Gag; GenBank Accession No. NM_002295) as template and using SEQ ID NO: 54 (Gag-F: 5'-GTCTGAATCTGATGGGTGCGAGAGCGTCAGTA-3') and SEQ ID NO: 55 (Gag-R: 5'-GTCGGATCCTTATTGTGACGAGGGGTCGTT-3') as primers. The resultant PCR product was inserted at the XhoI/BamHI site of the pTMD-mRFP-C3 vector.

TMD-mRFP-LR was prepared as follows. PCR was carried out using a pET28a-TEV-LR vector (LR; GenBank Accession No. NM_002295) as template and using SEQ ID NO: 56 (LR-F: 5'-GTCTGAATCTGATGTCCGGAGCCCTTGATGT-3') and SEQ ID NO: 57 (LR-R: 5'-GTCGGATCCTTAAGACCAGTCAGTGGTTGCTC-3') as primers. The resultant PCR product was inserted at the XhoI/BamHI site of the pTMD-mRFP-C3 vector.

The second construct AzG-KRS was prepared as follows. PCR was carried out using pET28a (GenBank Accession No. NM_005548) as template and using SEQ ID NO: 58 (KRS-F: 5'-GTCTGAATCTGATGGCGGCCGTGCAGGCG-3') and SEQ ID NO: 59 (KRS-R: 5'-GTCCCCGGGCTAGACAGAAGTGCCAAGTGTGTG-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pAzG-C3 vector.

Thus prepared first construct and second construct were transformed into HEK293 cell line. Fluorescence was observed as in the same manner as in Example <4-1> except for using DMEM.

As seen in FIG. 11, all the three proteins of the first construct were observed to move toward the cell membrane (left panel). As for p38 (see FIG. 11 A), which is known to bind to KRS, the second construct bound to KRS moved toward the cell membrane. However, such translocation was not observed for the potential binding proteins Gag (see FIG. 11 B) and LR (see FIG. 11 C) (central panel). Accordingly, it was verified that KRS protein binds with p38 protein but not with Gag or LR protein. Further, it was verified that AzG fluorescent protein may be used instead of EGFP.

<4-3> Analysis of Binding Between RelA Protein and Inhibitor Protein Ikb in Cell in Real Time

It is known that the interactions of NFkB and Ikb proteins regulated by TNF-alpha are involved with various cell signaling pathways occurring in cells. Of the various cell signaling pathways, particularly noticeable is the close relationship with inflammatory responses against various harmful signals in body. Accordingly, in order to verify the binding of the multifunctional inflammation-related gene RelA and the inhibitor protein Ikb, among the NFkB complexes, and to demonstrate the possibility of direct analysis in living cells after external stimulation, not by cell fixation, experiments were carried out using a real-time (time-laps) technique.

To this end, a first construct (TMD-mRFP-RelA) and a second construct (EGFP-Ikb) were prepared as follows.

The first construct TMD-mRFP-RelA was prepared as follows. PCR was carried out using a pEYFP-RelA vector (RelA; GenBank Accession No. NM_021975) as template and using SEQ ID NO: 60 (RelA-F: 5'-GGACTCGAGATGACGAAGTGTCCCCCTC-3') and SEQ ID NO: 61 (RelA-R: 5'-GAAGGATCCCGTTAGGAGCTGATCTGACTCAGCAGG-3') as primers. The resultant PCR product was inserted at the XhoI/BamHI site of the pTMD-mRFP-C3 vector.

The second construct EGFP-Ikb was prepared as follows. PCR was carried out using a pcDNA3-Ikb vector (Ikb; GenBank Accession No. NM_020529) as template and using SEQ ID NO: 62 (Ikb-F: 5'-GAAGAATTCTGATGTTCCAGGCGGCCGAGCG-3') and SEQ ID NO: 63 (Ikb-R: 5'-GAAGGATCCCGTCATAAACGTCAGCTGGCCTCCAA-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pEGFP-C3 vector.

Thus prepared first construct and second construct were transformed into CHO-k1 cell line and fluorescence was observed as in Example <4-1> at 0, 1, 2 and 3 minute.

As seen in FIG. 12, the first construct (TMD-mRFP-RelA) was uniformly distributed in the cell at 0 minute, but moved gradually toward the cell membrane with the lapse of time (left panel). The second construct (EGFP-Ikb) was also uniformly distributed in the cell at 0 minute, but moved gradually toward the cell membrane along with the first construct with the lapse of time (central panel). Especially, the constructs were observed to move toward the cell membrane from at about 10 seconds after the PMA treatment (not shown in the figure). The translocation was completed in most cells within 3 minutes. This tendency was observed similarly in all the preceding examples and all the following examples (not shown in the figure).

<4-4> Analysis of Binding of Multifunctional Inflammation-Related Gene NFkB Complex (RelA/p50/Ikb) in Cell

Basically, the NFkB complex is known to be formed by NFkB (RelA/p50) and the negative regulator Ikb protein. It

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was verified whether the binding of the three proteins RelA, p50 and IκB, which constitute the complex, can be identified.

To this end, a first construct (TMD-mRFP-RelA) and two second constructs (EGFP-IκB and HcR-p50) were prepared as follows. As the fluorescent label, mRFP was used for the first construct, and EGFP and HcR (HcRed), which is distinguishable to mRFP, were used for the second constructs.

The first construct (TMD-mRFP-RelA) and the IκB-comprising second construct (EGFP-IκB) were the same as those used in Example <4-3>. The other second construct HcR-p50 was prepared as follows. PCR was carried out using a pDMV-SPORT6-NFκB1 vector (NFκB1; GenBank Accession No. BC006231) as template and using SEQ ID NO: 64 (p50-F: 5'-GCTGAATTCTGATGGCAGAAGATGATCCATATT-3') and SEQ ID NO: 65 (p50-R: 5'-GCTCCCGGGCTTAATGCTTCATCCCAGCATTAGA-3') as primers. The resultant PCR product was inserted at the EcoRI/XmaI site of the pHcR-C3 vector.

Thus prepared first construct and two second constructs were transformed into HEK293 cell line, and fluorescence was observed as in Example <4-1>.

As seen in FIG. 13, all the first construct (TMD-mRFP-RelA, red), the second construct (EGFP-IκB, green) and the second construct (HcR-p50, blue) moved toward the cell membrane in response to the PMA stimulation. In contrast, the change of distribution was not observed in the control group (TMD-mRFP) wherein the first construct did not include a translocation module (not shown in the figure).

This experimental result indicates that not only the binding of the three protein complexes comprising the first construct, but also the binding of four complexes EGFP, mRFP, HcRed and BFP with the fluorescent label removed from the first construct can be verified using a generally used fluorescence microscope or confocal laser fluorescence microscope. Using various fluorescent protein species and the Meta (Carl Zeiss) or Spetral (Leica) fluorescence microscope, at least five protein complexes including BFP, CFP, GFP, RFP and Far-Red can be detected in addition to the first construct.

<4-5> Analysis of Binding of Multiple Interactions of Proteins (Lysyl RNA Synthetase (KRS), Arginyl RNA Synthetase (RRS), Asparagyl RNA Synthetase (DRS), p43 and p38)

In order to examine whether the method of the present invention could validate the complex binding of multiple proteins, p38 was inserted in the 1st construct attached a translocation module and each of KRS, RRS, DRS and p43 was inserted in the 2nd constructs, respectively. Thus prepared first construct and two second constructs were transformed into HEK293 cell line, and fluorescence was observed as in Example <4-1>.

As can be seen in FIG. 17, the inventors found that PMA treatments make fluorescence dye of RRS, DRS, p43 and p38 as well as KRS to move to a cell membrane, thereby binding to the inside of the cell.

Example 5

Verification of Candidate Materials Screened Through Y2H

Currently, Y2H is the most widely used for screening protein bindings in a living cell. It is also the typical method used for new drug screening. However, Y2H is disadvantageous in new drug targeting and verification because of the high false positive and the use of yeast. Accordingly, the applicability of the present invention as a method of verifying the candidate materials screened through Y2H was investigated.

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Four positive clones were screened through Y2H for super bacteria (antibiotics-resistant bacteria)-related OmpA (GenBank Accession No. AY485227) protein and human protein library (Entire library screening clones: 1.188×10^5 clones; first patch/streak screening: 137 clones; second re-transformation screening: 54 clones; third prey auto-activation test screening: 14 clones; fourth nucleotide sequencing confirmation: 4 clones).

Among the four positive clones, EEF1A1 (GenBank Accession No. BC009875), FAM14B (GenBank Accession No. BC015423) and DDX31 (GenBank Accession No. AK027002) were tested for binding with the OmpA protein. To this end, a first construct (TMD-mRFP-OmpA) and second constructs (EGFP-EEF1A, EGFP-FAM14B and EGFP-DDX31) were prepared as follows.

The first construct TMD-mRFP-OmpA was prepared as follows. PCR was carried out using a pET28a-OmpA (GenBank Accession No.: AY185227) vector as template and using SEQ ID NO: 66 (OmpA-F: 5'-GCTGAATTCTGATGAAATTGAGTCGTATTGCAC-3') and SEQ ID NO: 67 (OmpA-R: 5'-GCTGGATCCTTATTGAGCTGCTGCAGAGAGC-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pTMD-mRFP-C3 vector.

The second constructs were prepared as follows. For EGFP-EEF1A, a pOTB7-EEF1A (GenBank Accession No. BC009875) vector was used as template and SEQ ID NO: 68 (EEF1A-F: 5'-GCTGAATTCTGATGGGAAAGGAAAA-GACTCA-3') and SEQ ID NO: 69 (EEF1A-R: 5'-GCTG-GATCCCGCTATTTAGCCTTCTGAGCTT-3') were used as primers. For EGFP-FAM14B, a pCMV-SPORT6-FAM14B (GenBank Accession No.: BC015423) vector was used as template and SEQ ID NO: 70 (FAM14B-F: 5'-GTCGAAT-TCTGATGGGAAAGGAGAGTGGATGG-3') and SEQ ID NO: 71 (FAM14B-R: 5'-GTCCGATCCCGTCAGCTG-GAAGGGGTGAAC-3') were used as primers. For EGFP-DDX31, a pME18S-FL3-DDX31 vector was used as template and SEQ ID NO: 72 (DDX31-F: 5'-GTCGAATTCTGATGTTTTCTCCAAAGAAGCAT-3') and SEQ ID NO: 73 (DDX31-R: 5'-GTCCGATCCCGT-TAAACTTTCTGGGAAGTCTTG-3') were used as primers. After carrying out PCR, each PCR product was inserted at the EcoRI/BamHI site of the pEGFP-C3 vector.

Thus prepared first construct and second constructs were transformed into HEK293 cell line, and fluorescence was observed as in Example <4-1>.

As seen in FIG. 14, EEF1A1 (A) and FAM14B (B) did not move toward the cell membrane along with the OmpA-bound first construct (false positive). In contrast, DDX31 (C) moved toward the cell membrane along with the OmpA-bound first construct, and thus, was confirmed as positive. Therefore, it can be seen that the method of the present invention provides better accuracy than Y2H and enables reconfirmation of positive binding.

Example 6

Verification of Efficiency of Anticancer Drug Through Real-Time Analysis

p53 protein, known as a potential carcinogenic protein, is known to facilitate carcinogenesis by binding at least to mdm2 protein. Through researches, nutlin3 was confirmed as a potent anticancer drug capable of inhibiting it. In an in vitro binding inhibition experiment using Biacore's surface plasmon resonance (SPR) technology, a p53-mdm2 binding inhibition effect of 90% or more was attained when at least 1

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micromolar of nutlin3 was treated, and the median inhibitory concentration (IC50) was about 90 nanomolar (Vassilev, L. T. et. al., 2004, *Science* 303, 844-848).

The interaction between p53 (GenBank Accession No. NM_00546) and mdm2 and the binding inhibition by nutlin3 were verified in living human cell line. To this end, a first construct (TMD-mRFP-p53N) and a second construct (AzG-mdm2N) were prepared as follows.

The first construct TMD-mRFP-p53N was prepared as follows. PCR was carried out using a pGEX-4T1-p53N vector (p53; GenBank Accession No. NM_000546) as template and using SEQ ID NO: 74 (p53N-F: 5'-GTCGAATTCTCATG-GAGGAGCCGACATCAGAT-3') and SEQ ID NO: 75 (p53N-R: 5'-GTCGGATCCTCACACGGGGGAG-CAGCCT-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the first construct vector (pTMD-mRFP-C3 vector).

The second construct EGFP-mdm2N was prepared as follows. PCR was carried out using a pGEX-4T1-mdm2N vector (mdm2; GenBank Accession No. NM_002392) as template and using SEQ ID NO: 76 (mdm2N-F: 5'-GTCGAATTCTGATGTGCAATACCAACAT-GTCTGTACC-3') and SEQ ID NO: 77 (mdm2N-R: 5'-GTCGGATCCTCATACTACCAAGTTCCTGTAGAT-3') as primers. The resultant PCR product was inserted at the EcoRI/BamHI site of the pAzG-C3 vector.

Thus prepared first construct and second construct were transformed into HEK393 cell line, and fluorescence was observed as in Example <4-1>. Nutlin3 was treated at 0, 0.5, 1, 5, 10, 25, 50, 100 and 200 nM. As in Example <3-2>, after treating with nutlin3 for 5 minutes, the cells were fixed with 3.8% formaldehyde and observed using a confocal microscope (Carl Zeiss LSM 510). 200 cells exhibiting red fluorescence were randomly selected and the distribution of the red fluorescence at the cell membrane was measured for each concentration.

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As seen in FIG. 15, mdm2 (green, central panel) moved toward the cell membrane in a living cell along with p53 (red, left panel) in response to the external stimulation (PMA) (see FIG. 15 A), whereas a change in distribution was not observed when the inhibitor nutlin3 was treated (see FIG. 15 B). This indicates that mdm2 binds with p53 in the absence of nutlin3, but the binding of mdm2 and p53 is inhibited by nutlin3. This result shows that the present invention enables a direct analysis of protein interactions occurring in a living cell and may be useful as a tool for new drug development associated with the bindings.

Also, as seen in FIG. 16, by the result that 200 cells exhibiting red fluorescence were randomly selected and the distribution of the red fluorescence at the cell membrane was measured for each concentration, a binding inhibition effect of 95% or more was attained at 20 nanomolar (nM), whereas a binding inhibition effect of 90% or more was attained at 1 micromolar in an in vitro experiment for living human cells (HEK293) using recombinant proteins (Vassilev, L. T. et. al., 2004, *Science* 303, 844-848). Accordingly, it can be seen that the present invention provides an analysis accuracy of about 50 times that of the existing in vitro technique. In addition, this result indicates that the present invention provides a technique capable of avoiding the risk of research for cells or body and clinical tests for humans, by the in vitro inhibitory result.

As described, the present invention provides a method enabling real-time detection of bindings and interactions of materials occurring in a living cell and a method for screening a material altering such interactions. The method of the present invention overcomes the disadvantages including inaccuracy and complexity of existing biomaterial interaction detection techniques, including in vitro method (in vitro and biochemical techniques), antibody binding (antibody precipitation) techniques, FRET, Bi-FC and FCS techniques, etc. By labeling both constructs to promote accuracy, the present invention provides a novel real-time, antibody-free analysis.

SEQUENCE LISTING

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Lys Ala Arg Leu Ser Tyr Ser Asp Lys Asn Leu Ile Asp Ser Met Asp
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Phe	Gln	Thr	Lys	Asp	His	Leu	Phe	Phe	Val	Met	Glu	Phe	Leu	Asn	Gly
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Gly	Asp	Leu	Met	Tyr	His	Ile	Gln	Asp	Lys	Gly	Arg	Phe	Glu	Leu	Tyr
	435						440					445			
Arg	Ala	Thr	Phe	Tyr	Ala	Ala	Glu	Ile	Met	Cys	Gly	Leu	Gln	Phe	Leu
	450					455					460				
His	Ser	Lys	Gly	Ile	Ile	Tyr	Arg	Asp	Leu	Lys	Leu	Asp	Asn	Val	Leu
465					470					475					480
Leu	Asp	Arg	Asp	Gly	His	Ile	Lys	Ile	Ala	Asp	Phe	Gly	Met	Cys	Lys
			485						490					495	
Glu	Asn	Ile	Phe	Gly	Glu	Ser	Arg	Ala	Ser	Thr	Phe	Cys	Gly	Thr	Pro
		500						505					510		
Asp	Tyr	Ile	Ala	Pro	Glu	Ile	Leu	Gln	Gly	Leu	Lys	Tyr	Thr	Phe	Ser
	515						520					525			
Val	Asp	Trp	Trp	Ser	Phe	Gly	Val	Leu	Leu	Tyr	Glu	Met	Leu	Ile	Gly
	530					535					540				
Gln	Ser	Pro	Phe	His	Gly	Asp	Asp	Glu	Asp	Glu	Leu	Phe	Glu	Ser	Ile
545					550					555					560
Arg	Val	Asp	Thr	Pro	His	Tyr	Pro	Arg	Trp	Ile	Thr	Lys	Glu	Ser	Lys
			565						570					575	
Asp	Ile	Leu	Glu	Lys	Leu	Phe	Glu	Arg	Glu	Pro	Thr	Lys	Arg	Leu	Gly
		580						585					590		
Val	Thr	Gly	Asn	Ile	Lys	Ile	His	Pro	Phe	Phe	Lys	Thr	Ile	Asn	Trp
	595						600					605			
Thr	Leu	Leu	Glu	Lys	Arg	Arg	Leu	Glu	Pro	Pro	Phe	Arg	Pro	Lys	Val
	610					615						620			

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Lys Ser Pro Arg Asp Tyr Ser Asn Phe Asp Gln Glu Phe Leu Asn Glu
625 630 635 640

Lys Ala Arg Leu Ser Tyr Ser Asp Lys Asn Leu Ile Asp Ser Met Asp
645 650 655

Gln Ser Ala Phe Ala Gly Phe Ser Phe Val Asn Pro Lys Phe Glu His
660 665 670

Leu Leu Glu Asp
675

<210> SEQ ID NO 8

<211> LENGTH: 2028

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Human Protein kinase C mutant, TMD

<400> SEQUENCE: 8

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gacgaggcga accagccctt ctgtgccgtg aagatgaagg aggcgctcag cacagagcgt      120
gggaaaacac tgggtgcagaa gaagccgacc atgtatcctg agtggaagtc gacgttcgac      180
gcccacatct atgagggggcg cgtcatccag attgtgctaa tgcgggcagc agaggagcca      240
gtgtctgagg tgaccgtggg tgtgtcggtg ctggccgagc gctgcaagaa gaacaatggc      300
aaggctgagt tctggctgga cctgcagcct caggccaagg tgttgatgtc tgttcagtat      360
ttcctggagg acgtggattg caaacagtct atgcgcagtg aggacgaggc caagttccca      420
acgatgaacc gcccgggagc catcaaacag gccaaaatcc actacatcaa gaaccatgag      480
tttatcgcca cttcttttgg gcaaccacc ttctgttctg tgtgcaaaga ctttgtctgg      540
ggcctcaaca agcaaggcta caaatgcagg caatgtaacg ctgccatcca caagaaatgc      600
atcgacaaga tcatcggcag atgcactggc accgcggcca acagccggga cactatattc      660
cagaaagaac gcttcaacat cgacatgccg caccgcttca aggttcacaa ctacatgagc      720
cccaccttct gtgaccactg cggcagcctg ctctggggac tggtgaaagca gggattaaag      780
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ggagttgctg gggaggacat gcaagacaac agtgggacct acggcaagat ctgggagggc     1020
agcagcaagt gcaacatcaa caacttcate ttccacaagg tcctgggcaa aggcagcttc     1080
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gaccacctgt tctttgtgat ggagttcctc aacggggggg acctgatgta ccacatccag     1320
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ggggagagcc gggccagcac cttctgcggc acccctgact atatgcccc tgagatccta     1560
cagggcctga agtacacatt ctctgtggac tgggtgtctt tcgggttctt tctgtacgag     1620
atgctcattg gccagtcccc cttccatggg gatgatgagg atgaactctt cgagtccatc     1680

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cgtgtggaca cgccacatta tccccgtgg atcaccaagg agtccaagga catcctggag 1740
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cccttcttca agaccataaa ctggactctg ctggaaaagc ggaggttgga gccacccttc 1860
agggcccaaag tgaagtcacc cagagactac agtaactttg accaggagtt cctgaacgag 1920
aaggcgcgcc tctcctacag cgacaagaac ctcctcgact ccatggacca gtctgcattc 1980
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<210> SEQ ID NO 9
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Labeling material, Enhanced Green Fluorescent
Protein

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<400> SEQUENCE: 9

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1      5      10     15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20     25     30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35     40     45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50     55     60
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65     70     75     80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85     90     95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100    105    110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115    120    125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130    135    140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145    150    155    160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165    170    175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180    185    190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
195    200    205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210    215    220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225    230    235

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<210> SEQ ID NO 10
<211> LENGTH: 716
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Labeling material for Enhanced Green
Fluorescent Protein

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<400> SEQUENCE: 10

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gcgacgtaaa cggccacaag ttcagcgtgt ccggcgaggg cgagggcgat gccacctacg	120
gcaagctgac cctgaagttc atctgcacca cgggcaagct gcccggtgcc tggcccaccc	180
tcgtgaccac cctgacctac ggcgtgcagt gcttcagccg ctaccccgac cacatgaagc	240
agcacgactt cttcaagtcc gccatgcccg aaggctacgt ccaggagcgc accatcttct	300
tcaaggacga cggcaactac aagaccgcg cgaggtgaa gttcgagggc gacacctg	360
tgaaccgcat cgagctgaag ggcctcgact tcaaggagga cggcaacatc ctggggcaca	420
agctggagta caactacaac agccacaacg tctatatcat ggccgacaag cagaagaacg	480
gcataagggt gaacttcaag atccgccaca acatcgagga cggcagcgtg cagctcgccg	540
accactacca gcagaacacc cccatcggcg acggccccgt gctgctgccc gacaaccact	600
acctgagcac ccagtcgcc ctgagcaaag accccaacga gaagcgcgat cacatggtcc	660
tgctggaggt cgtgaccgcc gccgggatca ctctcgcat ggacgagctg tacaag	716

<210> SEQ ID NO 11

<211> LENGTH: 225

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Labeling material, Monomeric Red Fluorescent Protein

<400> SEQUENCE: 11

Met Ala Ser Ser Glu Asp Val Ile Lys Glu Phe Met Arg Phe Lys Val	
1 5 10 15	
Arg Met Glu Gly Ser Val Asn Gly His Glu Phe Glu Ile Glu Gly Glu	
20 25 30	
Gly Glu Gly Arg Pro Tyr Glu Gly Thr Gln Thr Ala Lys Leu Lys Val	
35 40 45	
Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp Ile Leu Ser Pro Gln	
50 55 60	
Phe Gln Tyr Gly Ser Lys Ala Tyr Val Lys His Pro Ala Asp Ile Pro	
65 70 75 80	
Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val	
85 90 95	
Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser	
100 105 110	
Leu Gln Asp Gly Glu Phe Ile Tyr Lys Val Lys Leu Arg Gly Thr Asn	
115 120 125	
Phe Pro Ser Asp Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp Glu	
130 135 140	
Ala Ser Thr Glu Arg Met Tyr Pro Glu Asp Gly Ala Leu Lys Gly Glu	
145 150 155 160	
Ile Lys Met Arg Leu Lys Leu Lys Asp Gly Gly His Tyr Asp Ala Glu	
165 170 175	
Val Lys Thr Thr Tyr Met Ala Lys Lys Pro Val Gln Leu Pro Gly Ala	
180 185 190	
Tyr Lys Thr Asp Ile Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr	
195 200 205	
Thr Ile Val Glu Gln Tyr Glu Arg Ala Glu Gly Arg His Ser Thr Gly	
210 215 220	

Ala
225

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<210> SEQ ID NO 12
<211> LENGTH: 674
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Labeling material, Monomeric Red Fluorescent
      Protein

<400> SEQUENCE: 12

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ccgtgaacgg ccacgagttc gagatcgagg gcgagggcga gggccgcccc tacgagggca      120
cccagaccgc caagctgaag gtgaccaagg ggggccccct gcccttcgcc tgggacatcc      180
tgtccctca gttccagtac ggctccaagg cctacgtgaa gcaccccgcc gacatccccg      240
actacttgaa gctgtccttc ccgaggggct tcaagtggga gcgcgtgatg aacttcgagg      300
acggcgggcgt ggtgaccgtg acccaggact cctccctgca ggacggcgag ttcatttaca      360
aggtgaagct gcgcggcacc aacttccctt ccgacggccc cgtaatgcag aagaagacca      420
tgggctggga ggctccacc gagcggatgt acccagagga cggcgccctg aagggcgaga      480
tcaagatgag gctgaagctg aaggacggcg gccactacga cgccgaggtc aagaccacct      540
acatggccaa gaagcccgtg cagctgcccg gcgcctacaa gaccgacatc aagctggaca      600
tcacctccca caacgaggac tacaccatcg tggaacagta cgagcgcgcc gagggcgccc      660
actccaccgg cgcc                                          674

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<210> SEQ ID NO 13
<211> LENGTH: 229
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Labeling material, Azami Green

<400> SEQUENCE: 13

Met Asp Pro Met Val Ser Val Ile Lys Pro Glu Met Lys Ile Lys Leu
 1             5             10            15

Cys Met Arg Gly Thr Val Asn Gly His Asn Phe Val Ile Glu Gly Glu
      20            25            30

Gly Lys Gly Asn Pro Tyr Glu Gly Thr Gln Ile Leu Asp Leu Asn Val
      35            40            45

Thr Glu Gly Ala Pro Leu Pro Phe Ala Tyr Asp Ile Leu Thr Thr Val
      50            55            60

Phe Gln Tyr Gly Asn Arg Ala Phe Thr Lys Tyr Pro Ala Asp Ile Gln
      65            70            75            80

Asp Tyr Phe Lys Gln Thr Phe Pro Glu Gly Tyr His Trp Glu Arg Ser
      85            90            95

Met Thr Tyr Glu Asp Gln Gly Ile Cys Thr Ala Thr Ser Asn Ile Ser
      100           105           110

Met Arg Gly Asp Cys Phe Phe Tyr Asp Ile Arg Phe Asp Gly Thr Asn
      115           120           125

Phe Pro Pro Asn Gly Pro Val Met Gln Lys Lys Thr Leu Lys Trp Glu
      130           135           140

Pro Ser Thr Glu Lys Met Tyr Val Glu Asp Gly Val Leu Lys Gly Asp
      145           150           155           160

Val Asn Met Arg Leu Leu Leu Glu Gly Gly Gly His Tyr Arg Cys Asp
      165           170           175

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Phe Lys Thr Thr Tyr Lys Ala Lys Lys Glu Val Arg Leu Pro Asp Ala
 180 185 190

His Lys Ile Asp His Arg Ile Glu Ile Leu Lys His Asp Lys Asp Tyr
 195 200 205

Asn Lys Val Lys Leu Tyr Glu Asn Ala Val Ala Arg Tyr Ser Met Leu
 210 215 220

Pro Ser Gln Ala Lys
 225

<210> SEQ ID NO 14
 <211> LENGTH: 687
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Labeling material, Azami Green

<400> SEQUENCE: 14

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actgtaaaacg ggcataatct cgtgattgaa ggagaaggaa aaggaaatcc ttacgagggg	120
acgcagattt tagacctgaa cgtcactgaa ggcgcacctc tgcctttcgc ttacgatatc	180
ttgacaacag tgtttccagta cggcaacagg gcattcacca agtaccacgc agatattcag	240
gactatttca agcagacttt tcctgagggg tatcactggg aaagaagcat gacttatgaa	300
gaccagggca tttgcaccgc cacaagcaac ataagcatga ggggcgactg tttttctat	360
gacattcggt ttgatggcac caactttcct cccaatgggc cggttatgca gaagaagact	420
cttaaatggg agccatccac tgagaaaatg tacgtagagg atggagtgtc gaagggtgat	480
gttaacatgc gcctgttgct tgaaggaggt ggccattatc gatgtgattt caaaactact	540
tacaaagcaa agaaggaggt ccgtttgcca gacgcgcaca aaattgacca ccgcattgag	600
atgttgaaagc atgacaaaga ttacaacaag gtcaagctct atgagaatgc cgttgctcgc	660
tattctatgc tgccgagtcg ggccaag	687

<210> SEQ ID NO 15
 <211> LENGTH: 228
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Labeling material, Heteractis crispa red
 fluorescent protein

<400> SEQUENCE: 15

Met Val Ser Gly Leu Leu Lys Glu Ser Met Arg Ile Lys Met Tyr Met	
1 5 10 15	
Glu Gly Thr Val Asn Gly His Tyr Phe Lys Cys Glu Gly Glu Gly Asp	
20 25 30	
Gly Asn Pro Phe Ala Gly Thr Gln Ser Met Arg Ile His Val Thr Glu	
35 40 45	
Gly Ala Pro Leu Pro Phe Ala Phe Asp Ile Leu Ala Pro Cys Cys Glu	
50 55 60	
Tyr Gly Ser Arg Thr Phe Val His His Thr Ala Glu Ile Pro Asp Phe	
65 70 75 80	
Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp Glu Arg Thr Thr Thr	
85 90 95	
Tyr Glu Asp Gly Gly Ile Leu Thr Ala His Gln Asp Thr Ser Leu Glu	
100 105 110	
Gly Asn Cys Leu Ile Tyr Lys Val Lys Val His Gly Thr Asn Phe Pro	

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<220> FEATURE:
 <223> OTHER INFORMATION: Nuclear Localization Signal (NLS) for construct

<400> SEQUENCE: 18

ggctctgggtg atgaagtcga aggagtggaa gaagtagcta agaagaagag taaaaaggaa 60
 aaggataaaa 69

<210> SEQ ID NO 19
 <211> LENGTH: 18
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Nuclear export Signal(NES) for construct

<400> SEQUENCE: 19

Asp Gln Arg Val Ile Ile Lys Leu Asn Ala His Val Gly Asn Ile Ser
 1 5 10 15

Leu Val

<210> SEQ ID NO 20
 <211> LENGTH: 54
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Nuclear export Signal(NES) for construct

<400> SEQUENCE: 20

gaccagcgcg tcacatcaaa gctgaacgcc catgtgggaa acatttcctt ggtg 54

<210> SEQ ID NO 21
 <211> LENGTH: 4727
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: pEGFP-C3 vector-GenBank Accession No. U57607

<400> SEQUENCE: 21

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 cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt 120
 gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca 180
 atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc 240
 aagtagcccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgccagta 300
 catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac 360
 catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg 420
 atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg 480
 ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggag gtaggcgtgt 540
 acggtgggag gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
 ccggtcgcca ccatggtgag caagggcgag gagctgttca ccggggtggt gcccatcctg 660
 gtcgagctgg acggcgacgt aaacggccac aagttcagcg tgcgcggcga gggcgagggc 720
 gatgccacct acggcaagct gaccctgaag ttcactctga ccaccggcaa gctgcccgtg 780
 ccctggccca ccctcgtgac caccctgacc tacggcgtgc agtgcttcag ccgctacccc 840
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 cgcaccatct tcttcaagga cgacggcaac tacaagaccc gcgccgaggt gaagttcgag 960
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aagcagaaga	acggcatcaa	ggtgaacttc	aagatccgcc	acaacatcga	ggacggcagc	1140
gtgcagctcg	ccgaccacta	ccagcagAAC	accccccacg	gcgacggccc	cgtgctgctg	1200
cccgacaacc	actacctgag	cacccagtcc	gccctgagca	aagaccccaa	cgagaagcgc	1260
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tcacaaattt	cacaaataaa	gcattttttt	cactgcattc	tagttgtggt	ttgtccaaac	1620
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aatttttgtt	aatcagctc	attttttaac	caataggccg	aaatcgcaa	aatcccttat	1740
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ccaggtgtgg	aaagtcccca	ggctccccag	caggcagaag	tatgcaaagc	atgoatctca	2400
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gatccggcta	cctgccatt	cgaccaccaa	gcgaaacatc	gcacgagcg	agcacgtact	3060
cggatggaag	ccggtcttgt	cgatcaggat	gatctggacg	aagagcatca	ggggctcgcg	3120
ccagccgaac	tgctccgag	gctcaaggcg	agcatgccc	acggcgagga	tctcgctgtg	3180
acccatggcg	atgcctgctt	gccgaatata	atggtggaaa	atggccgctt	ttctggattc	3240
atcgactgtg	gccggctggg	tgtggcgag	cgctatcagg	acatagcgtt	ggctaccctg	3300
gatattgctg	aagagcttgg	cggcgaatgg	gctgaccgct	tcctcgtgct	ttacggtatc	3360

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gccgctcccg attcgcagcg catcgccctc tatcgccctc ttgacgagtt cttctgagcg 3420
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<210> SEQ ID NO 24
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 <223> OTHER INFORMATION: Reverse primer for PRKCD

<400> SEQUENCE: 24

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for TMA

<400> SEQUENCE: 25

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<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for TMA

<400> SEQUENCE: 26

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<212> TYPE: DNA
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<400> SEQUENCE: 27

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<220> FEATURE:
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<400> SEQUENCE: 28

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<223> OTHER INFORMATION: Forward primer for PKCD

<400> SEQUENCE: 29

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<223> OTHER INFORMATION: Forward primer for Y313F

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<223> OTHER INFORMATION: Reverse primer for PKCD

<400> SEQUENCE: 32

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<223> OTHER INFORMATION: Forward primer 1 for NLS

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<400> SEQUENCE: 36

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<400> SEQUENCE: 37

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 <220> FEATURE:
 <223> OTHER INFORMATION: Forward primer 4 for NLS

 <400> SEQUENCE: 39

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 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Forward primer 1 for NES

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 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Reverse primer for NES

 <400> SEQUENCE: 41

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 <212> TYPE: DNA
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 <220> FEATURE:
 <223> OTHER INFORMATION: Forward primer 2 for NES

 <400> SEQUENCE: 42

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<210> SEQ ID NO 43
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 <400> SEQUENCE: 43

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<210> SEQ ID NO 44
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 <220> FEATURE:
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<400> SEQUENCE: 44

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Reverse primer for Azami Green

<400> SEQUENCE: 45

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<210> SEQ ID NO 46

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

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<223> OTHER INFORMATION: Forward primer for Heteractis crispa red fluorescent protein

<400> SEQUENCE: 46

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<210> SEQ ID NO 47

<211> LENGTH: 38

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Reverse primer for Heteractis crispa red fluorescent protein

<400> SEQUENCE: 47

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<211> LENGTH: 35

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<223> OTHER INFORMATION: Reverse primer for p53

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<223> OTHER INFORMATION: Forward primer for SV40T

<400> SEQUENCE: 50

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<400> SEQUENCE: 51

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<223> OTHER INFORMATION: Forward primer for p38

<400> SEQUENCE: 52

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<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for p38

<400> SEQUENCE: 53

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<223> OTHER INFORMATION: Forward primer for Gag

<400> SEQUENCE: 54

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<223> OTHER INFORMATION: Reverse primer for Gag

<400> SEQUENCE: 55

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<223> OTHER INFORMATION: Forward primer for LR

<400> SEQUENCE: 56

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<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for LR

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<400> SEQUENCE: 57

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<211> LENGTH: 29

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<220> FEATURE:

<223> OTHER INFORMATION: Forward primer for KRS

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<223> OTHER INFORMATION: Reverse primer for KRS

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<223> OTHER INFORMATION: Forward primer for RelA

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<220> FEATURE:

<223> OTHER INFORMATION: Reverse primer for RelA

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<223> OTHER INFORMATION: Forward primer for IkB

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<223> OTHER INFORMATION: Reverse primer for IkB

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<210> SEQ ID NO 64

<211> LENGTH: 33

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for p50

<400> SEQUENCE: 64

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<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for p50

<400> SEQUENCE: 65

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<210> SEQ ID NO 66
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for OmpA

<400> SEQUENCE: 66

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for OmpA

<400> SEQUENCE: 67

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<210> SEQ ID NO 68
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for EEF1A

<400> SEQUENCE: 68

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for EEF1A

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for FAM14B

<400> SEQUENCE: 71

gtcggatccc gtcagctgga agggggtgaa c 31

<210> SEQ ID NO 72
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<213> ORGANISM: Artificial Sequence
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gtcggatccc gttaaacttt ctgggaagtc ttg 33

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<223> OTHER INFORMATION: Forward primer for p53N

<400> SEQUENCE: 74

gtcgaattct catggaggag ccgcagtcag at 32

<210> SEQ ID NO 75
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for p53N

<400> SEQUENCE: 75

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<210> SEQ ID NO 76
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for mdm2N

<400> SEQUENCE: 76

gtcgaattct gatgtgcaat accaacaatgt ctgtacc 37

<210> SEQ ID NO 77
<211> LENGTH: 33
<212> TYPE: DNA
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<220> FEATURE:

<223> OTHER INFORMATION: Reverse primer for mdm2N

<400> SEQUENCE: 77

gtcggatcct catactacca agttcctgta gat

33

The invention claimed is:

1. A method for detecting interactions of a bait and a prey comprising the steps of:

- (a) preparing a cell comprising
 - (i) a first construct comprising a bait, a first labeling material and a translocation module; and
 - (ii) a second construct comprising a prey and a second labeling material;
- (b) allowing the bait of the first construct and the prey of the second construct to interact;
- (c) treating with a signaling material to translocate the first construct to the plasma membrane of the cell; and
- (d) detecting the translocation of the first construct and the second construct to the plasma membrane of the cell in the cell, wherein the translocation module is protein kinase C or its variants, wherein protein kinase C or its variants comprise an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7.

2. The method of claim 1, wherein the first labeling material is selected from the group consisting of GFP (Green Fluorescent Protein); EGFP (Enhanced Green Fluorescent Protein); RFP (Red Fluorescent Protein); mRFP (Monomeric Red Fluorescent Protein); DsRed (*Discosoma* sp. red fluorescent protein); CFP (Cyan Fluorescent Protein); CGFP (Cyan Green Fluorescent Protein); YFP (Yellow Fluorescent Protein); AzG (Azami Green), HcR (HcRed, *Heteractis crispa* red fluorescent protein), and BFP (Blue Fluorescent Protein).

3. The method of claim 1, wherein the second labeling material is selected from the group consisting of GFP (Green Fluorescent Protein); EGFP (Enhanced Green Fluorescent Protein); RFP (Red Fluorescent Protein); mRFP (Monomeric

10 Red Fluorescent Protein); DsRed (*Discosoma* sp. red fluorescent protein); CFP (Cyan Fluorescent Protein); CGFP (Cyan Green Fluorescent Protein); YFP (Yellow Fluorescent Protein); AzG (Azami Green), HcR (HcRed, *Heteractis crispa* red fluorescent protein), and BFP (Blue Fluorescent Protein).

15 4. A method for detecting interactions of a bait and a prey comprising the steps of:

- (a) preparing a cell comprising (i) a first construct comprising a bait, a first labeling material which is EGFP (Enhanced Green Fluorescent Protein) or mRFP (Monomeric Red Fluorescent Protein) and a translocation module having amino acid sequences of any one which is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7; and (ii) a second construct comprising a prey and a second labeling material of any one which is selected from the group consisting of EGFP (Enhanced Green Fluorescent Protein), mRFP (Monomeric Red Fluorescent Protein), AzG (Azami Green) and HcR (HcRed, *Heteractis crispa* red fluorescent protein);
- (b) allowing the bait of the first construct and the prey of the second construct to interact;
- (c) treating with a signaling material to translocate the first construct to the plasma membrane of the cell; and
- (d) detecting the translocation of the first construct and the second construct to the plasma membrane of the cell in the cell.

5. The method of claim 4, wherein the treatment with a signaling material is the treatment with 50 nM to 5 μ M of PMA (Phorbol 12-myristate 13-acetate, Phorbol ester).

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